(51) International Patent Classification 6 :		(11) International Publication Number:	WO 95/07992
C12N 15/60, 9/88, C07K 16/40, G01N 33/53, A61K 38/51, C12N 1/21, 5/20	A2	(43) International Publication Date:	23 March 1995 (23.03.95)

(21) International Application Number: PCT/US94/09478

(22) International Filing Date: 24 August 1994 (24.08.94)

(30) Priority Data:
08/123,859   17 September 1993 (17.09.93)   US

(71) Applicant: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 22nd floor, 300 Lakeside Drive, Oakland, CA 94612 (US).

(72) Inventors: TOBIN, Allan, J.; 850 South Masselin Avenue, Los Angeles, CA 90036 (US). ERLANDER, Mark, G.; 1352 Via Terrassa, Encinitas, CA 92024 (US). KAUFMAN, Daniel, L.; Apartment C, 1453 Centinela, Santa Monica, CA 90404 (US). CLARE-SALZLER, Michael, J.; 62112 N.W. 24th Lane, Gainesville, FL 32606 (US).

(74) Agents: BOSTICH, June, M. et al.; Spensley Horn Jubas & Lubitz, 5th floor, 1800 Century Park East, Los Angeles, CA 90067 (US).

(81) Designated States: AU, CA, JP, KR, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: CLONED GLUTAMIC ACID DECARBOXYLASE

(57) Abstract

Isolated polypeptides useful in ameliorating GAD-associated autoimmune disease as well as diagnostic and therapeutic methods of using the peptides are disclosed.

- 1 -

CLONED GLUTAMIC ACID DECARBOXYLASE

This application is a continuation-in-part
application of U.S. Serial No. 07/716,909, filed June
18, 1991 which is a continuation-in-part application of
U.S. Serial No. 07/586,536, filed September 21, 1990.

BACKGROUND OF THE INVENTION

The present invention was supported by Grant
NS22256 from the National Institutes of Health. The
United States Government has certain rights in this
invention.

1. FIELD OF THE INVENTION

The present invention relates to glutamic acid
decarboxylase$_{65}$ (GAD$_{65}$) polypeptides and methods of using
GAD$_{65}$ polypeptides diagnostically and therapeutically in
autoimmune disease.

2. DESCRIPTION OF THE BACKGROUND ART

Insulin-dependent diabetes mellitus (IDDM; type I
diabetes) is one of the most common metabolic
disorders. In the United States, IDDM affects
approximately one in 300 to 400 people, and
epidemiological studies suggest that its incidence is
increasing. The disease results from the autoimmune
destruction of the insulin-producing β-cells of the
pancreas. More specifically, the preonset stage is
characterized by "insulitis", in which lymphocytes
infiltrate the pancreatic islets and selectively
destroy the β-cells. Insulitis may be present for many
years before the onset of clinical symptoms. The
typical IDDM presentation of hyperglycemia appears only
after at least 80% of the insulin-producing β-cells are
lost. The remaining β-cells are destroyed during the
next few years.

Although insulin therapy allows most IDDM patients
to lead normal lives, this replacement is imp rfect and
does not complet ly restore metabolic homeostasis.
Thus, severe complications which result in dysfunctions

- 2 -

of the eye, kidney, heart, and other organs are common
in IDDM patients undergoing insulin therapy. Because
of this, it is highly desirable to extend the latency
period and prevent progression (e.g., through

5   administration of immunosuppressant drugs to interfere
with the autoimmune process and insulin to achieve
better control of the effects of sustained
hypoglycemia) between the start of β-cell destruction
and the actual requirement of insulin replacement

10 (i.e., when 80% of the β-cells are destroyed).
Therefore, a diagnostic test which determines the
beginning of β-cell destruction would allow the
clinician to administer immunosuppressant drugs
(Silverstein, et al., New England Journal of Medicine,

15 319:599-604, 1988) or prophylactic insulin therapy
(Keller, et al., Lancet, 341:927, 1993) to extend this
latency period and thus significantly delay the onset
of insulin replacement side effects.

Many IDDM patients have sera which contain

20 antibodies to a 64kD molecule (Baekkeskov, et al.,
J.Clin.Invest., 79:926-934, 1987; Atkinson, et al.,
Lancet, 335:1357-1360, 1990), to islet cell cytoplasmic
(ICA) molecules or islet cell surface (ICSA) molecules
(Bottazzo, et al, Lancet, 1:668-672, 1980), or to

25 insulin (Palmer, et al., Science, 222:1137-1139, 1983;
Atkinson, et al., Diabetes, 35:894-898, 1986).
Atkinson and coworkers (Atkinson, et al., Lancet,
335:1357-1360, 1990) have demonstrated that the
presence of antibodies to the 64kD molecule in human

30 sera appears to be the earliest and most reliable
indicator that onset of IDDM symptoms will eventually
occur.

Recently, Baekkeskov and coworkers established that
the 64kD molecule and glutamic acid decarboxylase (GAD)

35 have several antigenic epitopes in common and thus they
may be identical or very similar molecul s. Although
this identification is an important finding, the use of

- 3 -

this information as a diagnostic tool for predicting
IDDM is quite cumbersome and limited unless knowledge
of the molecular biology of GAD is known. Studies by
Kaufman, *et al.*, (*J. Clin. Invest.*, $\underline{89}$:283, 1992)
established that the 64kD molecule was intact GAD_{65}.
Consequently, the cloning and subsequent production of
large quantities of GAD_{65} or a GAD molecule which is
antigenically substantially identical to the GAD_{65}
molecule or fragments of the GAD_{65} molecule, both of
which can be easily purified, will allow the
development of a diagnostic kit designed to predict
IDDM as well as effective therapeutic modalities. The
present invention provides a means for accomplishing
these results.

SUMMARY OF THE INVENTION

The present invention arose out of the discovery
that recombinant DNA technology could be used to
produce eukaryotic GAD_{65} polypeptide and that GAD_{65}
polypeptide could be used in the diagnosis and therapy
of patients with autoimmune disease. Particularly
relevant is the use of eukaryotic GAD_{65} polypeptide in
the diagnosis and therapy of patients having, or at
risk of having, GAD-associated autoimmune disorders
such as insulin-dependent diabetes mellitus (IDDM) or
stiff man disease.

A major advantage of the present invention is that
it provides the art with a ready source of eukaryotic
GAD_{65} polypeptide corresponding to that purified from
natural sources, while avoiding the problems associated
with the isolation of naturally occurring eukaryotic
GAD_{65} polypeptide when separating it from other
eukaryotic non-GAD_{65} polypeptides. This absence of
other eukaryotic non-GAD_{65} polypeptides is significant
in that it allows the development of test systems which
will only detect antibodies specifically reactiv with
GAD_{65} polyp ptides.

- 4 -

Another advantage f providing eukaryotic GAD_{65}
polyp ptide in host cells is that by so doing, it is
possible to obtain much larger quantities of the
polypeptide than are currently practicably available
from natural sources. As a consequence, not only is it
possible to use the polypeptide of the invention to
more accurately classify and treat patients with such
autoimmune diseases as IDDM, but it is also now
possible to provide commercially useful quantities of
GAD polypeptide for use in diagnostic systems and
pharmaceutical compositions.

DESCRIPTION OF THE DRAWINGS

FIGURE 1   Cloning strategy for obtaining GAD_{65} and
GAD_{67} specific cDNA probes.

FIGURE 2   DNA sequence and corresponding amino
acid sequence for rat GAD_{65}.

FIGURE 3   DNA sequence and corresponding amino
acid sequence for human GAD_{65}.

FIGURE 4   Comparison of rat GAD_{65} and human GAD_{65}
amino acid sequences.

FIGURE 5 GAD_{65} and GAD_{67} cDNAs hybridize to
different size RNAs.

FIGURE 6   Southern blots hybridized with CDNA
probes specific for GAD_{65} and GAD_{67}.

FIGURE 7   Immunological identification of GAD_{65}
and GAD_{67}.

FIGURE 8   Proliferative T-cell responses of NOD
mice to β cell antigens.

FIGURE 9   Characterization of GAD specific T-cell
response of NOD mice as primed Th1 cells by enhanced
clonal size (a) and cell surface markers (b) and IFNγ
secretion.

FIGURE 10   Intramolecular spreading of T-cell
autoimmunity within the GAD molecule.

FIGURE 11   Delay of onset of IDDM following
immunization with GAD_{65}.

- 5 -

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the manipulation
of genetic materials by recombinant DNA procedures
which make possible the production of polypeptides
5   possessing part or all of the primary structural
conformation for one or more of the epitopes for
binding autoantibodies to glutamic acid decarboxylase$_{65}$
(GAD$_{65}$) and for polypeptides that bind to MHC receptors
to block T-cell recognition. These polypeptides are
10   highly useful for the immunological detection of
autoantibodies reactive with them, since such
autoantibodies are pre-diagnostic and indicative of
autoimmune diseases such as insulin dependent diabetes
mellitus and "stiff man" syndrome. These polypeptides
15   can also be used for purposes of screening drugs, such
as those that alter GAD function, and for generation of
polyclonal and monoclonal antibodies which, in turn,
can be used diagnostically to detect GAD$_{65}$.

The development of specific DNA sequences encoding
20   eukaryotic GAD$_{65}$ polypeptide for splicing into DNA
vectors can be accomplished using a variety of
techniques. For example, alternative methods which can
be employed include (1) the isolation of a double
stranded DNA sequence from the genomic DNA of the
25   eukaryote; (2) the chemical manufacture of a DNA
sequence to provide the necessary codons for the
polypeptide of interest; and (3) the in vitro synthesis
of a double stranded DNA sequence by reverse
transcription of mRNA isolated from a eukaryotic donor
30   cell. In the latter case, a double stranded DNA
complement of MRNA is eventually formed which is
generally referred to as CDNA.

The manufacture of DNA sequences is frequently the
method of choice when the entire sequence of amino acid
35   residues of the desired polypeptide product is known.
When th   ntire sequence f amino acid residues of th
d sired polypeptide is not known, the direct
manufacture of DNA sequences is not possible and the
method f choic is the formation of cDNA sequences.

- 6 -

Among the standard procedures for isolating cDNA
sequences of interest is the formation of plasmid-
carrying cDNA libraries which are derived from reverse
transcription of mRNA which is abundant in donor cells
5   that have a high level of genetic expression. When
used in combination with polymerase chain reaction
technology, even rare expression products can be
cloned. In those cases where significant portions of
the amino acid sequence of the polypeptide are known,
10   the production of labeled single or double stranded DNA
or RNA probe sequences duplicating a sequence
putatively present in the target cDNA may be employed
in DNA/DNA hybridization procedures which are carried
out on cloned copies of the cDNA which have been
15   denatured into a single stranded form (Jay, et al.,
Nucleic Acid Research, <u>11</u>:2325, 1983).

Hybridization procedures are useful for the
screening of recombinant clones by using labeled mixed
synthetic oligonucleotide probes wherein each is
20   potentially the complete complement of a specific DNA
sequence in the hybridization sample which includes a
heterogeneous mixture of denatured double stranded DNA.
For such screening, hybridization is preferably
performed on either single stranded DNA or denatured
25   double stranded DNA. These procedures are particularly
useful in the detection of cDNA clones derived from
sources where an extremely low amount of mRNA sequences
relating to the polypeptide of interest are present.
.In other words, by using stringent hybridization
30   conditions directed toward avoidance of non-specific
binding, it is possible, for example, to allow the
autoradiographic visualization of a specific cDNA clone
by the hybridization of the target DNA to that single
probe in the mixtur which is its complet complement
35   (Wallace, et al., *Nucleic Acid Research*, <u>9</u>:879, 1981).

In addition, a GAD cDNA library can be scre ned by
injecting the various cDNAs into oocytes, allowing
sufficient tim for xpression f th cDNA gene
products to occur, and testing for the presence of the

- 7 -

desired cDNA expression product, for example, by using
antibody specific for GAD$_{65}$ polypeptide, by using
functional assays for GAD$_{65}$ enzymatic activity, or by
measuring the ability of the expression product to
5   stimulate pathogenic T-cells.

Alternatively, a cDNA library can be screened
indirectly for GAD$_{65}$ peptides having at least one
epitope using antibodies to GAD$_{65}$ (Chang and Gottlieb,
J.Neurosci., 8:2123, 1988). Such antibodies can be
10   either polyclonally or monoclonally derived and used to
detect expression product indicative of the presence of
GAD$_{65}$ cDNA. Preferred are antibodies directed to an
epitope found in the first 100 amino acids of the N-
terminal portion of GAD$_{65}$.

15   Of the three above-noted methods for developing
specific DNA sequences for use in recombinant
procedures, the use of genomic DNA isolates, is the
least common. This is especially true when it is
desirable to obtain the microbial expression of
20   mammalian polypeptides because of the presence of
introns.

The present invention provides novel polypeptides
of GAD$_{65}$ which have part or all of the primary
structural conformation, that is, a continuous sequence
25   of amino acid residues, having at least one epitope for
antibodies to GAD$_{65}$ or at least one determinant for T-
cell recognition. It is possible to use the
polypeptide fragments of the invention rather than
intact GAD to detect autoantibodies to GAD. The term
30   "polypeptide," as applied to GAD polypeptide, includes
any sequence of amino acids having an epitope for
autoantibodies to GAD or binds to a T-cell MHC
receptor. Thus, the polypeptide fragments of GAD
encompassed by the invention possess a biological
35   activity such as the ability to induce and/or bind
autoantibodies to GAD, bind to T-cell MHC receptors
(especially receptors on pathogenic T-cells) and the
like.

- 8 -

The polypeptides resulting from microbial
expression of the DNA sequenc s of the invention or
from other synthetic techniques, such as solid-phase
peptide synthesis, can be further characterized by
5   their freedom from association with other eukaryotic
polypeptides or other contaminants which might
otherwise be associated with GAD in its natural
cellular environment or in such extracellular fluids as
plasma or urine.

10   Studies by the present inventors unequivocally
establish that GAD_{65} and GAD_{67} are encoded by distinct
genes and are not produced, for example, by
post-transcriptional or post-translational modification
of a common genomic sequence. Evidence proving that
15   GAD_{65} and GAD_{67} are encoded by different genes include:
(a) the largest contiguous sequence of exact identity
between GAD_{65} and GAD_{67} cDNAs is only 17 nucleotides in
length, (b) cDNAs from GAD_{65} and GAD_{67} do not cross
hybridize with each other's or with each other's mRNA
20   under low stringency conditions (2.0 x SSC, 0.01% SDS,
23°C), and (c) GAD_{65} and GAD_{67} cDNAs do not cross
hybridize with isolated genomic clones encoding GAD_{67}
and GAD_{65}, respectively.

The term "host" includes not only prokaryotes, but
25   also such eukaryotes as yeast, filamentous fungi, plant
and animal cells, as well as insect cells which can
replicate and express an intron-free DNA sequence of
eukaryotic GAD_{65}. However, prokaryotes are preferred as
the host organism for screening purposes while
30   eukaryotic cells, especially insect cells, are
preferred for expression.

The term "prokaryotes" includes all bacteria which
can be transformed or transfected with the gene for the
xpression of GAD_{65}. Prokaryotic hosts may include gram
35   negative as well as gram positive bacteria such as, for
example, *E. coli, S. typhimurium, Serratia marc scens
and Bacillus subtilis*.

- 9 -

A recombinant DNA molecule coding for the GAD$_{65}$ polypeptides can be used to transform or transfect the host using any of the techniques commonly known to those of ordinary skill in the art. Especially
5 preferred is the use of a plasmid or a virus containing the GAD$_{65}$ coding sequence for purposes of prokaryotic transformation or transfection, respectively. Alternatively, liposomes containing the DNA of interest can be used to obtain expression in the host (Zhu, et
10 al., *Science*, 261:209, 1993)

Methods for preparing fused, operably linked genes and expressing them in bacteria are well-known in the art (Maniatis, *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring
15 Harbor, NY, 1989). The genetic constructs and methods described therein can be utilized for expression of GAD$_{65}$ in prokaryotic hosts.

In general, expression vectors containing promotor sequences which facilitate the efficient transcription
20 of the inserted eukaryotic genetic sequence are used in connection with the host. The expression vector typically contains an origin of replication, a promoter, and a terminator, as well as specific genes which are capable of providing phenotypic selection of
25 the transformed cells. The transformed prokaryotic hosts can be grown in fermentors and cultured according to techniques known in the art to achieve optimal cell growth. The polypeptides of the invention can then be isolated from the grown medium, cellular lysates, or
30 cellular membrane fractions.

The isolation and purification of the expressed polypeptides of the invention may be by any conventional means such as, for example, preparative chr mat graphic separations and immunological
35 separations such as those involving the use of m n clonal or polyclonal antibody.

By having provided the sequence of amino acid residues of GAD$_{65}$, the present invention provides for the manufacture of DNA sequences which code for the

- 10 -

host expression of polypeptide analogs or derivatives of GAD$_{65}$ which differ from naturally-occurring forms in terms of the identity or location of one or more amino acid residues and which share some or all of the

5   epitopes of naturally-occurring polypeptide forms.

The novel DNA sequences of the invention include all sequences useful in providing the expression in prokaryotic or eukaryotic host cells of polypeptides which have at least a part of the primary structural

10   conformation for one or more epitopes capable of reacting with autoantibodies to GAD$_{65}$ which are comprehended by: (a) the DNA sequence as set forth in Figures 2 or 3 or their complementary strands; (b) DNA sequences which hybridize to DNA sequences defined in

15   (a) or fragments thereof; and (c) DNA sequences which, but for the degeneracy of the genetic code, would hybridize to DNA sequences defined in (a) and (b) above. Specifically comprehended in (b) are genomic DNA sequences which encode allelic variant forms of

20   GAD$_{65}$. Part (c) specifically comprehends the manufacture of DNA sequences which encode GAD$_{65}$, GAD$_{65}$ fragments, and GAD$_{65}$ analogs wherein the DNA sequences thereof may incorporate codons which facilitate translation of mRNA in non-vertebrate hosts.

25   Since the cDNA sequence of the invention encodes essentially the entire human or rat GAD$_{65}$ molecule, it is now a matter of routine to prepare, subclone, and express smaller polypeptide fragments of cDNA from this or a corresponding cDNA sequence which would encode as

30   few as one epitope for autoantibodies to human or rat GAD$_{65}$. The presence of such an epitope on a cloned polypeptide can then be confirmed using, for example, serum from a patient with autoantibodies to GAD$_{65}$. An exampl of such a smaller peptide is the first

35   approximately 100 amino acids from the N-terminus of GAD$_{65}$ (shown in Figure 3). This amino acid sequence is essentially absent from GAD$_{67}$. Other examples of specific peptides of the invention are shown in Table 7

- 11 -

as well as the approximate carboxy-terminal two-thirds
of GAD from about amino acid 224 to about amino acid
585. Especially preferred in the carboxy-terminal two-
thirds of GAD is the amino acid segment from about
5   amino acid 224 to about amino acid 398.

The present invention further relates to monoclonal
antibodies which are specific for the polypeptides of
the invention as well as the diagnostic and therapeutic
use of these monoclonal antibodies. This specificity
10   enables the monoclonal antibody, and like monoclonal
antibodies with like specificity, to be used to bind
the polypeptide of the invention when the polypeptide,
or amino acids comprising the polypeptide, are present
in specimens or a host, such as a human.

15   Numerous techniques can be utilized to produce the
monoclonal antibodies of the invention without
resorting to undue experimentation. To a great extent,
the products of such monoclonal antibodies is rendered
routine because of the highly defined nature of the
20   polypeptides of the invention. Thus, whether the
polypeptides of the invention are used for immunization
and/or screening, the very limited number of
immunogenic determinants on the polypeptides greatly
simplifies the identification of cell lines producing
25   monoclonal antibodies of the invention, for example, by
limiting the repertoire of clonal expression possible.

One very useful type of cell line for expression of
the monoclonal antibodies of the invention is the
hybridoma. The general method used for production of
30   hybridomas producing monoclonal antibody is well known
(Kohler and Milstein, *Nature*, 256:495, 1975). The
resulting hybridomas were then screened for production
of monoclonal antibodies capable of binding to the
polypeptides of the invention.

35   The techniques of sensitization and/or
immunization, cell fusion, ascites production,
selecti n of mixed hybridomas, or subcloning of
monoclonal hybridomas are generally well known in the
art. Attention is direct d to Koprowski, et *al*., U.S.

- 12 -

Patent No. 4,172,124, Koprowski, *et al.*, U.S. Patent
No. 4,196,265, or Douillard, J.Y. and Hoffman, T.,
Basic Facts about Hybridomas, in *Compendium of
Immunology*, Vol. II, L. Schwartz, ed. (1981), which are
5   herein incorporated by reference. In general, the
purified peptides can be modified to have a cystine
attached at the C-terminus to permit unidirectional
attachment of the synthetic peptide to an immunogenic
protein through a connecting bridge, for example,
10 maleimidobenzoylated (MB)-keyhole limpet hemocyanin
(KLH). Other immunogenic conjugates can also be used,
for example, albumin, and the like. The resulting
structure may have several peptide structures linked to
one molecule of protein.
15   Somatic cells derived from a host immunized against
the synthetic peptides can be obtained by any suitable
immunization technique. The host subject is immunized
by administering the antigen, usually in the form of a
protein conjugate, as indicated above, by any suitable
20 method, preferably by injection, either
intraperitoneally, intravenously, subcutaneously, or by
intra-foot pad. Adjuvants may be included in the
immunization protocol.
        The initial immunization with the protein bound
25 antigen can be followed by several booster injections
given periodically at intervals of several weeks. The
antibody contained in the plasma of each host can then
be tested for its reactivity with the immunizing
polypeptide of the invention. The host having the
30 highest response is usually most desirable as the donor
of the antibody secreting somatic cells used in the
production of hybridomas. Alternatively,
hyperimmunization can be effected by repeatedly
injecting additional amounts of peptide-protein
35 conjugate by intravenous and/or intraperiton al route.

- 13 -

The isolation of hybridomas producing monoclonal
antibodies of the invention can be accomplished using
routine screening techniques which permit determination
of the elementary reaction pattern of the monoclonal
5   antibody of interest. Thus, if a monoclonal antibody
being tested binds with a polypeptide of the invention,
then the antibody being tested and the antibody
produced by the hybridomas of the invention are
equivalent.

10   Alternatively, since the invention teaches
polypeptides or amino acid sequences which are
specifically required for binding of the preferred
monoclonal antibodies of the invention, it is now
possible to use these peptides for purposes of

15   immunization to produce hybridomas which, in turn,
produce monoclonal antibodies specific for the
polypeptide. This approach has the added advantage of
decreasing the repertoire of monoclonal antibodies
generated by limiting the number of antigenic

20   determinants presented at immunization by the
polypeptide. The monoclonal antibodies produced by
this method can be screened for specificity using
standard techniques, for example, by binding
polypeptide to a microtiter plate and measuring binding

25   of the monoclonal antibody by an ELISA assay.

It is also possible to determine, without undue
experimentation, if a monoclonal antibody has the same
specificity as a monoclonal antibody of the invention
by ascertaining whether the former prevents the latter

30   from binding the polypeptide of the invention. If the
monoclonal antibody being tested competes with the
monoclonal antibody of the invention, as shown by a
decrease in binding by the monoclonal antibody of the
invention, then it is likely that the two monoclonal

35   antibodies bind to the same, or a closely related,
epitope.

Still another way to determine whether a monoclonal
antibody has the sp cificity of a monoclonal antibody
of the invention is to pr -incubate the monoclonal

- 14 -

antibody of the invention with the polypeptide of the
invention with which it is normally reactive, and then
add the monoclonal antibody being tested to determine
if the monoclonal antibody being tested is inhibited in
5   its ability to bind the antigen. If the monoclonal
antibody being tested is inhibited then, in all
likelihood, it has the same, or a closely related,
epitopic specificity as the monoclonal antibody of the
invention.

10   The GAD_{65} of the invention is particularly suited
for use in immunoassays in which it can be utilized in
liquid phase or bound to a solid phase carrier. In
addition, GAD_{65} used in these assays can be detectably
labeled in various ways.

15   Examples of immunoassays which can utilize the GAD_{65}
of the invention are competitive and non-competitive
immunoassays in either a direct or indirect format.
Examples of such immunoassays are the radioimmunoassay
(RIA), the sandwich (immunometric assay) and the
20   Western blot assay. Detection of antibodies which bind
to the GAD_{65} of the invention can be done utilizing
immunoassays which run in either the forward, reverse,
or simultaneous modes, including immunohistochemical
assays on physiological samples. The concentration of
25   GAD_{65} which is used will vary depending on the type of
immunoassay and nature of the detectable label which is
used. However, regardless of the type of immunoassay
which is used, the concentration of GAD_{65} utilized can
be readily determined by one of ordinary skill in the
30   art using routine experimentation.

The GAD and GAD fragments of the invention can be
bound to many different carriers and used to detect the
presence of antibody specifically reactive with the
polyp ptide. Alternatively, the carrier-bound GAD and
35   GAD fragments can be used therapeutically for
extracorporeal absorption of autoimmune antibodies in
patients having, r at risk of having, GAD-associated
disorders. Examples of well-known carriers include
glass, polystyren , polyvinyl chloride, polypropylene,

- 15 -

polyethylene, p lycarbonate, dextran, nylon, amyloses,
natural and modified celluloses, polyacrylamides,
agaroses, and magnetite. The nature of the carrier can
be either soluble or insoluble for purposes of the

5   invention. Those skilled in the art will know of other
suitable carriers for binding GAD_{65}, or will be able to
ascertain such, using routine experimentation.

There are many different labels and methods of
labeling known to those of ordinary skill in the art.

10   Examples of the types of labels which can be used in
the present invention include enzymes, radioisotopes,
colloidal metals, fluorescent compounds,
chemiluminescent compounds, and bioluminescent
compounds.

15   Alternatively, the polypeptide of the invention
which comprises the GAD enzymatic domain can be used to
detect antibodies to GAD by measuring GAD enzymatic
activity. For example, GAD_{65} and a specimen suspected
of having antibodies to GAD_{65} can be incubated for a

20   period of time and under conditions sufficient to allow
binding to occur between GAD_{65} and the antibodies. The
reaction product is precipitated and then tested for
GAD enzymatic activity.

For purposes of the invention, the antibody which

25   binds to GAD_{65} of the invention may be present in
various biological fluids and tissues. Any sample
containing a detectable amount of antibodies to GAD_{65}
can be used. Normally, a sample is a liquid such as
urine, saliva, cerebrospinal fluid, blood, serum and

30   the like, or a solid or semi-solid such as tissue,
feces and the like.

The materials for use in the assay of the invention
are ideally suited for the preparation of a kit. Such
a kit may comprise a carrier means being

35   compartmentaliz d to receive in close confinement one
or mor container m ans such as vials, tubes and the
like, ach of the container means comprising one of the
separat lements to be used in the method. For
example, one of the container means may comprise GAD_{65}

- 16 -

bound to a carrier. A second container may comprise
soluble, detectably-labeled second antibody, in
lyophilized form or in solution.

In addition, the carrier means may also contain a
5   plurality of containers each of which comprises
different, predetermined amounts of GAD_{65}. These latter
containers can then be used to prepare a standard curve
into which can be interpolated the results obtained
from the sample containing the unknown amount of
10   autoantibodies to GAD_{65}.

In using the kit all the user has to do is add, to
a container, a premeasured amount of a sample
containing a measurable, yet unknown amount of
autoantibodies to GAD_{65} to be detected, a premeasured
15   amount of carrier-bound GAD_{65} present in the first
container, and a premeasured amount of the detectably
labeled second antibody present in the second
container. Alternatively, the non-detectably labeled
GAD_{65} can be provided attached to the container to which
20   the sample and the detectably labeled second antibody
are added. After an appropriate time for incubation,
an immune complex is formed and is separated from the
supernatant fluid, and the immune complex or the
supernatant fluid are detected, as by radioactive
25   counting or addition of an enzyme substrate, and color
development.

In an alternative embodiment, a kit comprising the
GAD polypeptide of the invention can be used to detect
·the stage of GAD-associated autoimmune disease in a
30   patient. As further shown herein, Applicants have
discovered that certain GAD peptides or fragments are
associated with different levels of progression in the
autoimmune disease and that the level of disease
process can be ascertained by looking at immune cell
35   proliferative response, such as that of the pathogenic
T-c 11 of the patient.

The term "ameliorate" denotes a lessening of the
detrimental effect of the autoimmune response in the
patient receiving therapy. The term "therapeutically

- 17 -

effective" means that the amount of GAD_{65} polypeptide
used is of sufficient quantity to ameliorate the cause
of disease due to the autoimmune response.

The GAD_{65} polypeptides, including whole GAD_{65}, of the
5   invention can be used therapeutically in patients
having, or at risk of having, an autoimmune response
associated with GAD_{65}. Such therapy can be
accomplished, for example, by the administration of
GAD_{65} polypeptide to induce tolerance to GAD. Such
10   administration can utilize unlabeled as well as labeled
GAD_{65} polypeptide. When unlabeled GAD_{65} polypeptide is
utilized advantageously, it would be in a form wherein,
for example, the GAD_{65} polypeptides are in fragments
which are too small to stimulate an immune response,
15   but large enough to bind, or block, the continuance of
the autoimmune response. For example, GAD_{65} could be
digested enzymatically into epitope-sized peptides
(typically 5-12 amino acids in length) and thereby bind
to Fab binding portions present in the body fluids, or
20   on the surface of immune cells, of the patient with
autoimmune disease. Alternatively, peptides having at
least one determinant for binding to T-cell MHC
receptor can be similarly produced or chemically
synthesized.

25   Alternatively, the GAD_{65} polypeptides of the
invention can be administered labeled with a
therapeutic agent. These agents can be coupled either
directly or indirectly to the GAD_{65} polypeptides of the
invention. One example of indirect coupling is by use
30   of a spacer moiety. These spacer moieties, in turn,
can be either insoluble or soluble (Diener, et al.,
Science, 231:148, 1986) and can be selected to enable
drug release from the GAD_{65} polypeptide at the target
site. Exampl s of therapeutic agents which can be
35   coupled to the GAD_{65} polypeptides of the invention for
immunotherapy are drugs, radioisotopes, lectins, and
toxins.

- 18 -

The drugs with which can be conjugated to the GAD_{65}
polypeptides of the invention include compounds which
are classically referred to as drugs such as mitomycin
C, daunorubicin, and vinblastine.

5   In using radioisotopically conjugated GAD_{65}
polypeptides of the invention for immunotherapy,
certain isotopes may be more preferable than others
depending on such factors as leukocyte distribution as
well as stability and emission. Depending on the

10   autoimmune response, some emitters may be preferable to
others. In general, α and β particle-emitting
radioisotopes are preferred in immunotherapy.
Preferred are short range, high energy α emitters such
as ^{212}Bi. Examples of radioisotopes which can be bound

15   to the GAD_{65} polypeptides of the invention for
therapeutic purposes are ^{125}I, ^{131}I, ^{90}Y, ^{67}Cu, ^{212}Bi, ^{211}At,
^{212}Pb, ^{47}Sc, ^{109}Pd and ^{188}Re.

Lectins are proteins, usually isolated from plant
material, which bind to specific sugar moieties. Many

20   lectins are also able to agglutinate cells and
stimulate lymphocytes. However, ricin is a toxic
lectin which has been used immunotherapeutically. This
is accomplished by binding the α-peptide chain of
ricin, which is responsible for toxicity, to the

25   antibody molecule to enable site specific delivery of
the toxic effect.

Toxins are poisonous substances produced by plants,
animals, or microorganisms that, in sufficient dose,
are often lethal. Diphtheria toxin is a substance

30   produced by *Corynebacterium diphtheria* which can be
used therapeutically. This toxin consists of an α and
β subunit which under proper conditions can be
separated. The toxic A component can be bound to GAD_{65}
polyp ptide and used for site specific delivery to a

35   leukocyte expressing a receptor for GAD_{65} polypeptide.

Other therapeutic agents which can be c upled to
the GAD_{65} polypeptides of the invention, as well as *ex
vivo* and *in vivo* therapeutic protocols, are known, or

- 19 -

can be easily ascertained, by those of ordinary skill
in the art.

The present invention also relates to a polypeptide
which can be administered therapeutically to
5 ameliorate, or utilized diagnostically to identify, the
disease process in patients having, or at risk of
having, this disease. The conventional single-letter
code used to represent the various amino acids relates
as follows:

10   **TABLE 1**

     Phe: F Leu: L Ile: I Met: M
     Val: V Ser: S Pro: P Thr: T
     Ala: A Tyr: Y His: H Gln: Q
15   Asn: N Lys: K Asp: D Glu: E
     Cys: C Trp: W Arg: R Gly: G

A polypeptide sequence of the invention was
identified by comparing the amino acid sequences of
20 human GAD_{65}, human GAD_{67}, and the P2-C protein of the
picornavirus, coxsackie virus. The P2-C polynucleotide
plays a role in the virus membrane bound replication
complex. These analyses established the presence of an
extensive sequence similarity between both GAD_{65}
25 molecules and the coxsackie virus. A core polypeptide
of six contiguous amino acid residues of the GAD_{65} and
P2-C polypeptide are identical in amino acid sequence.
Indeed, of the 24 amino acids in the polypeptide, 19
·are identical or conserved. In addition, there also
30 exists a high charge density and the presence of a
proline residue which would render this region highly
antigenic (see Table 2).

TABLE 2

COMPARISON OF AMINO ACID SEQUENCES

Protein	Amino Acid Sequence
Human GAD$_{67}$	$_{258}$S I M A A R Y K Y F P E V K T K G M A A V P K L$_{281}$
Human GAD$_{65}$	$_{250}$A M M I A R F K M F P E V K E K G M A A L P R L$_{273}$
Coxsackie Virus P2-C	$_{28}$F I E W L K V K I L P E V K E K H E F - L S R L$_{50}$

The solid line encloses identical amino acids whereas the dashed line encloses amino acid residues with similar charge, polarity, or hydrophobicity.

- 21 -

In Table 2, the solid line ncloses identical amino acids whereas the dashed line encloses amino acid residues with similar charge, polarity, or hydrophobicity.

5   The discovery of this common polypeptide region supports an etiologic role for "molecular mimicry" in the precipitation of diabetes. Thus, where a patient genetically susceptible to IDDM is infected by a coxsackie virus, the immune response to the similar GAD
10   sequence in the patient's β-cells. The immunological response is maintained by the antigenically similar GAD polypeptides resulting in the eventual destruction of the β-cells and the subsequent presentation of IDDM.

At present, it is believed that the destruction of
15   pancreatic β-cells in IDDM is mediated by a cellular autoimmune response. As described herein, a polypeptide of the invention can ameliorate the autoimmune response to GAD. Because of the complexity of autoimmune disease, it is possible to envision
20   numerous possible therapeutic modalities which would allow the polypeptides of the invention to be used to ameliorate such diseases. In one embodiment, it appears that the polypeptides of the invention can be utilized to block recognition by a specific T cell
25   receptor (TCR) or an MHC receptor presenting an autoimmune antigen on the surface of an antigen presenting cell (APC). The inhibition of such recognition might occur, for example, by providing the patient with the polypeptide of the invention which, in
30   turn, can displace the autoimmune antigen being presented in the antigen-cleft of the MHC receptor. However, although not wanting to be bound to a particular theory, it is believed that the polypeptides of the invention probably act to induce or restore a
35   tolerogenic stat by direct interaction with the appropriat TCR on the surface of a GAD specific pathogenic T-cell. This latter therapeutic appr ach of

- 22 -

direct interaction with the TCR is supported by the
examples and suggests that suppression of the
autoimmune response can be achieved through induction
of high-zone tolerance by use of high concentrations of
5   polypeptide, preferably soluble. Another possible
mechanism is that the polypeptide of the invention may
play a role in anergizing pathogenic T cells by binding
to the T cell MHC receptor, thereby preventing the
appropriate costimulatory signal.

10   Alternatively, the polypeptides of the invention
could be used to stimulate a T-suppressor cell
population in order to restore self-recognition and,
thereby, ameliorate the autoimmune disease.
Stimulation of T-suppressor cell populations could be
15   achieved, for example, by use of a bi-specific antibody
having one variable region specific for an epitope
present on the autoimmune antigen residing in the cleft
of the MHCII receptor and, a second variable region
specific for an epitope present on the CD8$^+$ receptor.
20   The production of antibody specific for the polypeptide
of the invention is a matter of routine to those of
skill in the art, as is the preparation of bi-specific
antibodies having specificity for 2 or more epitopes.

        Polypeptide analogs of the present invention may be
25   designed which will compete for recognition of self-
antigens at the level of antigen presentation or induce
anergy in T cells, due to a lack of a costimulatory
signal. Since MHC molecules contain a single peptide
binding site, it is possible to design polypeptides
30   which will bind with high affinity to disease-
associated MHC molecules, but will not activate
disease-causing T-helper cells. Such polypeptides act
as antagonists for self-antigen recognition. In the
present invention, support for this mechanism is found
35   in the examples, especially Example 7. Precedent for
such an approach aris s from observation that a mouse
lysozyme polypeptide, its lf non-immunogenic, can

- 23 -

compete for MHC binding with an immunogenic polypeptide
from hen-egg white lysozyme and thereby reduce T cell
activation by that polypeptide (Adorini, et al.,
Nature, 334:623-625, 1988) as well as studies using T-
5   cell receptor peptides to block formation of complex
between T-cells, autoantigen and MHC (Howell, et al.,
Science, 246:668, 1989). Similarly, such a therapeutic
approach for screening effective polypeptide analogs
has been utilized in such autoimmune diseases as
10   experimental autoimmune encephalomyelitis (EAE)
(Wraith, et al., Cell, 59:248, 1989; Urban, et al.,
Cell, 59:257, 1989).

        The single-letter symbols used to represent the
amino acid residues in the polypeptides of the present
15   invention are those symbols commonly used in the art.
The peptides of the invention include not only the
natural amino acid sequences, but also peptides which
are analogs, chemical derivatives, or salts thereof.
The term "analog" or "conservative variation" refers to
20   any polypeptide having a substantially identical amino
acid sequence to a polypeptide provided herein and in
which one or more amino acids have been substituted
with chemically similar amino acids. For example, one
polar amino acid, such as glycine or serine, may be
25   substituted for another polar amino acid; or one acidic
amino acid, such as aspartic acid may be substituted
for another acidic amino acid, such as glutamic acid;
or a basic amino acid, such as lysine, arginine, or
histidine may be substituted for another basic amino
30   acid; or a non-polar amino acid such as alanine,
leucine, or isoleucine may be substituted for another
non-polar amino acid.

        The term "analog" or "conservative variation" also
means any polypeptide which has one or more amino acids
35   deleted from or added to a polypeptide of the present
invention, but which still retains a substantial amino
acid sequence homology to such peptide. A substantial

- 24 -

sequence homology is any homology greater than 70%,
preferably at least about 80%, and more preferably at
least about 90%. The term "fragment" also means any
shorter version of the polypeptides identified herein
5   having at least 6 amino acid residues, wherein the
fragment possesses biological activity, or is a
fragment capable of inhibiting the stimulation of T-
cells by a stimulating polypeptide fragment or
substantially full-length molecule.

10   The term "chemical derivative" means any
polypeptide derived from a polypeptide of the present
invention and in which one or more amino acids have
been chemically derivatized by reaction of the
functional side groups of amino acid residues present
15   in the polypeptide. Thus, a "chemical derivative" is a
polypeptide that is derived from the sequences or
polypeptides identified herein by one or more chemical
steps. Such derivatized molecules include, for
example, those molecules in which free amino groups
20   have been derivatized to form amine hydrochlorides, P-
toluene sulfoamides, benzoxycarboamides, T-butyloxycar-
boamides, thiourethane-type derivatives, trifluoroacet-
ylamides, chloroaceamides, or formamides. Free carbox-
yl groups may be derivatized to form salts, methyl and
25   ethyl esters or other types of esters or hydrazides.
Free hydroxyl groups may be derivatized to form O-acyl
or O-alkyl derivatives. The imidazole nitrogen of
histidine may be derivatized to form N-im-benzylhis-
tidine. Also included as chemical derivatives are
30   those polypeptides which contain one or more naturally
occurring amino acids derivatives of the 20 standard
amino acids. For example, 4-hydroxyproline may be
substituted for proline; 5-hydroxylysine may be
substitut d for lysine; 3-methylhistidin may be
35   substituted for histidine; homoserine may be
substituted for serine, and ornithine may be
substituted for lysine.

- 25 -

It should be understood that the present invention is not limited to the illustrative polypeptides depicted in Table 2 and Table 9, instead, a polypeptide falling within the scope of this invention may extend

5   outside of, or comprise less than, the region between amino acid 28 and amino acid 50 of coxsackie virus P2-C, or between amino acid 250 and amino acid 273 of GAD_{65}, or between amino acid 258 and amino acid 281 of GAD_{67}, as well as the region between amino acid 78 and

10   amino acid 97, or between amino acid 247 and amino acid 266, or between amino acid 335 and amino acid 356, or between amino acid 479 and amino acid 498, or between amino acid 509 and amino acid 528, or between amino acid 524 and amino acid 543, or between amino acid 539

15   and amino acid 556, or between amino acid 564 and amino acid 583 of GAD_{65}, as long as a substantial part of a given polypeptide is characterized by an amino acid sequence from that region, or segments or combinations thereof, and the polypeptide demonstrates the desired

20   immunological or biological activity against autoimmune disease. In addition, polypeptides according to this invention include those having amino acid sequences which are longer or shorter in length than those of the polypeptides illustrated in Table 2 and Table 9, or

25   which comprise segments or combinations thereof, as long as such polypeptides consist substantially of the region between the amino acids illustrated in Table 2 and Table 9 and demonstrate immunological or biological activity. All polypeptides of the invention should not

30   stimulate or enhance the autoimmune disease.

Accordingly, it should be understood that the specific selection of any one polypeptide within the polypeptides of the invention does not involve undue experimentation. Such a selection may be carried out

35   by taking a number of polypeptides and testing them for their immunological and biological activity in ameli rating the autoimmune disease or for detecting

- 26 -

antibody. The NOD mouse represents an excellent and well characterized model for screening polypeptides of the invention capable of ameliorating or preventing diabetes. Example 7 illustrates an acceptable

5   procedure for routine screening of candidate polypeptides with biologic activity.

The polypeptides according to the present invention may be prepared by recombinant techniques or by conventional synthesis using known polypeptide

10   synthetic methods, including synthesis on a solid support. An example of a suitable solid phase synthetic technique is that described by Merriweather (*J.Am.Chem.Soc.*, 85:2149, 1963). Other polypeptide synthetic techniques may be found, for example, in

15   Bodanszky, *et al.*, *Peptide Synthesis*, John Wiley & Sons, 2d ed., 1976, as well as other references known to those skilled in the art. A summary of polypeptide synthesis techniques can be found in Stewart, *et al.*, *Solid Phase Peptide Synthesis*, Pierce Chemical Company,

20   Inc., Rockford, Ill., 1984. The synthesis of polypeptides by solution methods may also be used, for example, as described in *The Proteins*, Vol. II, 3d ed., Neurath, *et al.*, eds., 105, Academic Press, New York, NY, 1976. Appropriate protective groups for use in

25   such synthesis can be found in the above references as well as in J. McOmie, *Protective Groups in Organic Chemistry*, Plenum Press, New York, NY, 1973.

The polypeptides of the invention may also be prepared in an appropriate host transformed with DNA

30   sequences that code for the desired polypeptide. For example, a polypeptide may be prepared by the fermentation of appropriate hosts that have been transformed with and which express a DNA sequence encoding th polyp ptide. Alternatively, a DNA

35   sequence encoding several of the polypeptides of this inv ntion may be linked tog ther and thos sequences may then be used to transform an appropriate host to

- 27 -

permit the expression of polypeptides involved in the autoimmune disease.

The dosage ranges for the administration of the GAD polypeptides of the invention are those large enough to produce the desired effect in which the symptoms or cellular destruction of the autoimmune response are ameliorated. The dosage should not be so large as to cause adverse side effects, such as unwanted cross-reactions, anaphylactic reactions, and the like. Generally, the dosage will vary with the age, condition, sex, and extent of the disease in the patient and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of any counterindications. Dosage can vary from about $0.1mg/m^2$ to about $2000mg/m^2$, preferably about $0.1mg/m^2$ to about $500mg/m^2/dose$, in one or more dose administrations daily, for one or several days.

The GAD polypeptides of the invention can be administered parenterally by injection or by gradual perfusion over time. The GAD polypeptides of the invention can be administered intravenously, intraperitoneally, intra-muscularly, subcutaneously, intracavity, transdermally, intranasally, or enterally.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenish rs (such as those based on Ringer's dex-

- 28 -

trose), and the like. Preservatives and other
additives may also be pres nt such as, for example,
antimicrobials, anti-oxidants, chelating agents, and
inert gases and the like.

5   The invention also relates to a method for
preparing a medicament or pharmaceutical composition
comprising the GAD$_{65}$ polypeptides of the invention, the
medicament being used for therapy of autoimmune
response to GAD$_{65}$.

10   The above disclosure generally describes the
present invention. A more complete understanding can
be obtained by reference to the following specific
examples which are provided herein for purposes of
illustration only and are not intended to limit the

15   scope of the invention.

EXAMPLE 1
CLONING AND EXPRESSION OF GAD$_{65}$

20   A.   RECOMBINANT DNA PROCEDURES
         In order to obtain cDNA probes specific for GAD$_{65}$
and GAD$_{67}$, total RNA was extracted from adult rat brain
by guanidine isothiocyanate-cesium gradient using the
method of Chirgwin, et al. (Biochemistry, 18:5294,

25   1979). Poly (A) RNA was purified on oligo dT
cellulose, using the protocol by Bethesda Research
Laboratories (BRL). First strand synthesis was
performed by using MMLV-reverse transcriptase (BRL),
with conditions suggested, except that poly d(N$_6$)-mers

30   (Pharmacia) were used as primers. This cDNA-RNA
mixture was heat inactivated at 65°C for 15 min and
stored at -20°C. For PCR, 1/50 of the sample was added
to the 100μl reaction. Degenerate oligonucleotides
were synthesized (Applied Biosystems) to encode the

35   underlined common amino acid sequ nces of feline (from
cDNA) (Kobayashi, et al., J.Neurosci., 7:2768, 1987)
and rat (from peptides) (Chang and Gottlieb,

- 29 -

J.Neurosci., 8:2123, 1988) GAD (Figure 1). The 5'-end
sequence of each degenerate oligonucleotide contained
one strand of the DNA sequence recognized by either
SstI and HindIII (5' oligo) or SstI and SstII (3'-end
5   oligo). These primers were used for selective
amplification by polymerase chain reaction of the
generated cDNA template as described by Gould, *et al.*
(*Proc.Natl.Acad.Sci.,USA*, 86:1934, 1989). PCR products
were subcloned into HindIII/SstI double digested
10   Bluescript SK vector (Stratagene), transformed into DH5
(BRL), and plated by standard methods (Maniatis, et
al., Molecular Cloning: A Laboratory Manual, Cold
Spring Harbor Laboratory, Cold Spring Harbor, NY,
1989).

15   Colony hybridization was done with an 5'-^{32}P end
labeled oligonucleotide specific to feline GAD$_{67}$
(Kobayashi, *et al.*, *J.Neurosci.*, 7:2768, 1987). End
labeling of oligonucleotide, hybridization conditions,
and washing conditions were done as described (Wallace,
20   et *al.*, in *Guide to Molecular Cloning Techniques*;
Berger, *et al.*, Eds. in *Methods of Enzymology*; Abelson,
et *al.*, Eds. *Academic Press, Inc.*, San Diego, 432-442,
1987), except that the nitrocellulose filters were
washed at 50°C for 15 min. Colonies which were
25   positive and negative in the hybridization were
individually picked and grown overnight in Terrific
Broth (Tartof, *et al.*, *Focus,* 9:12, 1987). DNA was
isolated using a boiling method (Maniatis, *et al.*,
Molecular Cloning: A Laboratory Manual, Cold Spring
30   Harbor Laboratory, Cold Spring Harbor, NY, 1989) and
templates were denatured by 0.2N NaOH and purified by
Sephacryl S400 spun columns (Pharmacia). Sequencing of
denatured double stranded template was by the
chain-t rmination method (Sanger, *et al.*,
35   *Proc.Natl.Acad.Sci.,USA*, 74:5463, 1977) using the
T7-sequencing kit (Pharmacia).

- 30 -

As shown in Figure 1, PCR-generated rat GAD_{65} and GAD_{67} cDNAs were used as probes to screen a lambda ZAP (Stratagene) rat hippocampus library provided by S. Heinemann (Salk Institute) by standard techniques
5   (Maniatis, et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989). A 2400 nucleotide GAD_{65} cDNA (the largest clone) was isolated and subcloned by "zapping" as described by Stratagene. When a rat GAD_{67} cDNA was
10   obtained which was smaller than a 3.2kb rat GAD_{67} cDNA clone already on hand, the larger cDNA was sequenced. Exo III deletions (Henikoff, *Gene*, 28:351, 1984) were made in both directions for GAD_{65} and GAD_{67} and templates were prepared and sequenced as described above.
15   Anchored PCR (Frohman, et al., *Proc.Natl.Acad.Sci.,USA*, 85:8998, 1988) was done to clone the remaining 5'-ends of GAD_{65} and GAD_{67} mRNAs which were not represented in the original cDNA clones isolated in the library screening. Sequencing of these clones revealed that
20   neither GAD_{65} nor GAD_{67} mRNAs contained any further initiation codons (AUGs) in frame with the previously designated initiation codons of the original cDNA clones.

25   **EXAMPLE 2**
             CHARACTERIZATION OF CLONED GAD_{65}

A.   NORTHERN BLOT HYBRIDIZATION
Two PCR-derived cDNA probes were hybridized to
30   Northern blots containing rat brain RNA in order to determine whether the GAD_{67} and GAD_{65} cDNAs were derived from two different mRNAs. RNA was extracted as described in Example 1. Poly (A) RNA was separated by electrophoresis in formaldehyde and transferred onto
35   Biotrans (ICN) membranes, and hybridization was performed as described by Well, et al. (*J.Neurosci.*, 16:311, 1986), except that 100µl/ml of poly (A) was

- 31 -

add d. Probes were labeled to approximately 10^9 dpm/μg
by the oligolabeling procedure of Feinberg and
Vogelstein (*Anal.Biochem.*, 132:6, 1983). Identical
results were subsequently obtained with full-length
5   clones of GAD$_{65}$ and GAD$_{67}$ cDNAs.

As shown in Figure 5, lanes 1 and 2 contain 1μg of
poly (A) selected RNA extracted from rat cerebellum.
Lane 1 was hybridized to a cDNA probe for the rat
cognate of feline GAD$_{67}$ (Kobayashi, *et al.*, *J.Neurosci.*,
10   7:2768, 1987) and lane 2 with a cDNA probe for the rat
peptide sequence (which corresponds to GAD$_{65}$).

The cDNA probe for the rat peptide sequence
hybridized to a 5.7kb RNA, while the cDNA probe for the
rat cognate of feline GAD$_{67}$ cDNA, hybridized to a 3.7kb
15   RNA. This demonstrates that GAD$_{65}$ and GAD$_{67}$ are not
derived from the same mRNA.

B. GENOMIC HYBRIDIZATION OF GAD$_{67}$AND GAD$_{65}$

In order to investigate the possibility that GAD$_{67}$
20   and GAD$_{65}$ arise from separate genes, cDNAs of both GAD$_{67}$
and GAD$_{65}$ were hybridized to DNA blots containing
genomic DNA.

For Southern blots, genomic DNA was extracted from
rat liver as described (Kaiser, *et al.*, in *DNA Cloning*,
25   vol.I, A Practical Approach, D.M. Glover ed., IRL
Press, Oxford, pp. 38-40, 1985). DNA (10μg/sample) was
digested to completion with EcoRI and HindIII using
conditions recommended by the suppliers (BRL,
Gaithersburg, MD). DNA fragments were separated by
30   electrophoresis at 1.5v/cm for 16 hrs in 0.8% agarose.
The DNA was then transferred to Zeta-Probe membranes
(Bio-Rad), hybridized, and washed, as described by
Gatti, *et al.* (*Biotechniques*, 2:148, 1984), except that
5μg/ml Carnation dried milk was substituted for
35   Denhardt's solution. Probes for Southern blots were
labeled as d scribed in Example 1, above.

- 32 -

As shown in Figure 6, genomic DNA digested with
HindIII and EcoRI are in lanes 1 and 3 and lanes 2 and
4, respectively. GAD$_{67}$ cDNA was hybridized to lanes 1
and 2, whereas GAD$_{65}$ cDNA was hybridized to lanes 3 and
5   4. Numbers along the side of the gel are the DNA
fragment sizes in kilobases.

This data shows that the two cDNAs hybridize to
genomic fragments of different sizes. In addition, the
greatest contiguous stretch of identical nucleotide
10   sequence of GAD$_{65}$ and GAD$_{67}$ cDNAs is only 17 nucleotide
bases in length. Thus, GAD$_{67}$ and GAD$_{65}$ are encoded by
two distinct genes.

C. ENZYMATIC COMPARISON OF GAD$_{67}$ AND GAD$_{65}$

15   Studies were done comparing the effect of PLP on
the activity of GAD$_{67}$ and GAD$_{65}$. In so doing, both cDNAs
were subcloned into vectors that allowed their
expression in bacteria (Studier, et al., J.Mol.Biol.,
189:113, 1986). Overexpression of "fusionless" GAD$_{65}$
20   and GAD$_{67}$ was accomplished by subcloning GAD$_{65}$ cDNA into
the NcoI site of pET-8c and GAD$_{67}$ cDNA into the NheI
site of pET-5c vectors (Studier, et al., J.Mol.Biol.,
189:113, 1986).

To obtain compatible sticky ends for correct in-
25   frame subcloning of both cDNAs, selective amplification
was performed by PCR using conditions suggested by
United States Biochemical (USB), with 200μM dNTPs and
1.5mM MgCl$_2$ in the mixture and annealing at 55°C with 20
cycles to decrease infidelity of AmpliTAQ (USB).
30   Primers specific for GAD$_{65}$ and GAD$_{67}$ contained one DNA
strand of the NcoI and SpeI recognition sites,
respectively. Since there is a NheI restriction site
within the coding region of GAD$_{67}$, SpeI (which is
compatible with NheI) was used.

35   PCR products were subcloned into their respective
pET vectors, transformed into DH5 and plated as
described (Maniatis, et al., Molecular Cloning: A

- 33 -

Laboratory Manual, Cold Spring Harbor Laboratory, Cold
Spring Harbor, NY, 1989). Colonies were picked and
grown overnight in LB broth with 50μg/ml ampicillin.
Subclones with correct orientation were transformed
5   into BL21(DE3) strain (Studier, *et al., J.Mol.Biol.,*
__189__:113, 1986) for overexpression. As a negative
control, the pET-8C vector with no insert was
transformed and subsequently induced. Single colonies
were picked, grown, induced by 1mM isopropyl-B-D-
10   thiogalacto-pyranoside (IPTG), and analyzed on SDS-PAGE
gels as described (Sambrook, *et al., Molecular Cloning
a Laboratory Manual*, Cold Spring Harbor Laboratory
Press, Cold Spring Harbor, 17.15-17.16, 1989).

To measure GAD activity, we induced 10ml cultures
15   of bacteria at OD_{600}-0.5 with 1mM IPTG. Two hours after
induction, bacteria was spun down and resuspended and
sonicated in 1ml of homogenizing buffer (1mM
phenylmethylsulfonyl fluoride (PMSF), 1mM 2-
aminoethylisothiouronium bromide (AET), and 60mM
20   potassium phosphate, pH 7.1). After sonication, cell
debris was removed by centrifugation and protein
concentration was measured (Bradford, *Anal.Biochem.,*
__72__:248, 1986) in the supernatant (supernatant was
stored in aliquots at -70°C). Brain homogenates were
25   prepared as described (Legay, *et al., J.Neurochem.,*
__46__:1478, 1986). GAD activity was measured as described
(Krieger, *et al., J.Neurochem.,* __33__:299, 1984) with
0.2mM PLP present or absent and 20μl of brain
homogenate or bacterial lysate in the incubation
30   mixture. Production of $^{14}CO_2$ in bacterial lysates was
linear with respect to time of incubation and protein
concentration.

- 34 -

TABLE 3

Source	GAD Specific Activity[a]		Fold Increase in Induction
	- PLP	+ PLP	
BL21(DE3) + pET-8c	12 ± 0.4	9 ± 1	--
BL21(DE3) + pET-GAD$_{65}$	115 ± 3	773 ± 61	6.7
BL21(DE3) + pET-GAD$_{67}$	160 ± 2	389 ± 8	2.4
Rat Brain	131 ± 5	216 ± 2	1.6

[a] cpms of $^{14}CO_2$/µg protein/hr of
triplicates ± S.E.M.

As shown in Table 3, bacterial lysates containing
GAD$_{65}$ or GAD$_{67}$ catalyze the conversion of [1-^{14}C]-
glutamate to GABA and $^{14}CO_2$.

PLP stimulates the enzymatic activity of GAD$_{65}$ more
than GAD$_{67}$. This greater stimulation probably reflects
the faster cycling of GAD$_{65}$ through the inactivation
cycle proposed by Martin and coworkers (Martin,
Cell.Mol.Neurobiol., 7:237, 1987). This faster cycling
suggests that GAD$_{65}$ contributes more to the pool of
apo-GAD that exists *in vivo* (Miller, *et al.*, *Brain
Res.Bull.*, 5(Suppl.2):89, 1980). Thus, *in vivo*, PLP
appears to regulate GAD$_{65}$ activity more than GAD$_{67}$
activity.

GAD$_{65}$ activity in bacterial lysates is similar to
the five-fold PLP stimulation of GAD activity found in
synaptosomes prepared from rat substantia nigra
(Miller, *et al.*, *J.Neurochem.*, 33:533, 1979). Because
both GADs are more dependent upon added PLP in bacteria
than is the GAD activity in crude rat brain
homogenates, the endogenous PLP concentration of
bacteria lysates may be less than rat brain
homogenates.

- 35 -

D.   IMMUNOL GICAL IDENTIFICATION OF GAD$_{65}$ AND GAD$_{67}$

Rat brain homogenates and bacterial lysates were
extracted as described above. Equal volumes of loading
buffer were added to each sample as described (Harlow,

5   et al., Antibodies, A Laboratory Manual, Cold Spring
Harbor Laboratory, Cold Spring Harbor, NY, 1988).
Proteins were separated by electrophoresis in a 10%
acrylamide gel in SDS and electrophoretically
transferred to nitrocellulose (Harlow, et al.,

10   Antibodies, A Laboratory Manual, Cold Spring Harbor
Laboratory, Cold Spring Harbor, NY, 1988). The
unreacted sites were blocked with a phosphate buffered
saline (PBS) solution containing 2% bovine serum
albumin (fraction V), 1% gelatin, and 1% Triton-X-100

15   at 42°C for one hr. After washing, the nitrocellulose
filter was then cut into three sections and incubated
with the following primary antibodies: lanes 1 to 4
with a 1/2000 dilution of the antiserum of Oertel, et
al. (Neuroscience, 6:2689, 1981), which recognizes both

20   GAD$_{67}$ and GAD$_{65}$; lanes 5-8 with a 1/2000 dilution of K-2
antiserum, which recognizes only GAD$_{67}$; lanes 9-12 with
a 1/2000 dilution of GAD-6 monoclonal antibody, which
is specific for GAD$_{65}$ (Chang, et al., J.Neurosci.,
8:2123, 1988). All filters were extensively washed and

25   appropriate secondary antibodies were incubated and
washed. Bound antibodies were detected with ^{125}I-labeled
protein A and autoradiography. Each lane contained the
following: lanes 1, 5, and 9 are BL21(DE3) + pET-GAD$_{67}$;
lanes 2, 6, and 10 are BL21(DE3) + pET-GAD$_{65}$; lanes 3,

30   7, and 11 are rat brain homogenate; and lanes 4, 8, and
12 are BL21(DE3) + pET-8c.

The immunoblots of bacterially produced GAD$_{65}$ and
GAD$_{67}$ demonstrated that GAD$_{65}$ indeed corresponds to the
smaller GAD in brain extracts, and GAD$_{67}$ to the larger

35   form (Figure 7). Previous work has demonstrated the

- 36 -

correspondence of GAD$_{67}$ to the larger GAD for feline
GAD$_{67}$, and for mouse GAD$_{67}$ (Katarova, et al.,
Eur.J.Neurosci., 2:190, 1990; 235, 1987). The
mobilities of bacterially produced GAD$_{65}$ and GAD$_{67}$ (as
5   detected with the antiserum of Oertel, et al.
(Neuroscience, 6:2689, 1981) are identical to the
immunoreactive doublet seen in rat brain homogenate.

The smaller molecular weight and larger molecular
weight forms of GAD in rat brain are thus identical in
10   antigenicity and size to the products of GAD$_{65}$ and GAD$_{67}$
cDNAs, respectively. Consequently, the two GADs in rat
brain are GAD$_{65}$ and GAD$_{67}$. From these data it can also
be concluded that the molecular identity of the
reported PLP-dependent and PLP-independent GADs by
15   Tapia (Bayon, et al., J.Neurochem., 29:519, 1977) are
GAD$_{65}$ and GAD$_{67}$, respectively. Martin and coworkers
(Spink, et al., Brain Res., 421:235, 1987) have
reported the existence of four kinetically different
forms of rat brain GAD. However, immunoblotting
20   experiments (with the antisera used here) of these
forms have not been reported.

E.   **DISTRIBUTION OF GAD$_{65}$ and GAD$_{67}$ IN RNAs IN BRAIN
TISSUE**

25   Experiments were done to determine the distribution
of GAD$_{65}$ and GAD$_{67}$ in RNAs in cerebellum using in situ
hybridization.

Transcripts of, respectively, 3.2kb and 2.3kb from
GAD$_{65}$ and GAD$_{67}$ cDNAs, were radiolabeled with ^{35}S
30   according to Wuenschell, et al.
(Proc.Natl.Acad.Sci.,USA, 83:6193, 1986) procedure.
Hydrolyzed fragments of 200 bp were hybridized to
coronal sections of a rat cerebellum. Animals were
anesthetized under halothane and decapitated. The
35   brain was rapidly frozen in dry ice and coronal frozen
secti ns (12μm) were fixed for 30 min in freshly
prepared 4% formaldehyde in phosphate-buffered saline

- 37 -

(PBS; 130mM NaCl, 10mM Na phosphate, pH 7.0). The
tissue was dehydrated through graded ethanol solutions
and stored at -70°C.

In order to increase tissue permeability, the
5   sections were submitted to the following pretreatments:
rehydration through graded ethanol solutions (5 min
each in 95%, 85%, 70%, 50%, and 30% ethanol); PBS (5
min); 0.02N HCl (10 min); PBS (5 min); 0.01% Triton N-
101 in PBS (1 min); PBS (2 x 5 min); 1μg/ml proteinase
10   K (7.5 min); and glycine (to inhibit proteinase K) in
PBS (3 x 5 min). Proteinase K was digested for 30 min
at 37°C before use. Sections were then incubated at
37°C in 50% formamide, 750mM NaCl, 25mM EDTA, 0.2% SDS,
0.02% BSA, 0.002% Ficoll, 0.02% polyvinylpyrrolidone,
15   250μg/ml yeast tRNA, 250μg/ml poly A, and 25mM PPES (pH
6.8).

For the hybridization, 100mM DTT, 10% dextran
sulfate, and sense or antisense ^{35}S-RNA were added to
the prehybridization solution. An aliquot (50μl) of
20   the hybridization solution containing about 3 ng (10^6
cpm) of probe (sense or antisense) was added onto the
slides. Each slide was coverslipped and incubated for
16 hrs at 50°C, following which the siliconized
coverslips were removed by brief washing in 4 x SSC (1
25   x SSC - 150mM NaCl, 60mM Na citrate, pH 7.0).

Sections were then treated with ribonuclease A
(50μg/ml in 0.5M NaCl, 10mM Na thiosulfate, 1mM EDTA,
10mM TrisHCL, pH 8.0) for 20 min at 37°C and rinsed for
2 hrs at room temperature in 2 x SSC, 10mM Na
30   thiosulfate, for 30 min at 55°C. Sections were
dehydrated in ethanol, delipidated in xylene, coated
with Kodak NTB2 emulsion and exposed for 10 days at
4°C. The emulsion was developed with Kodak D19, and
the tissu counterstained with cresyl violet.

35   Autoradiographic grains were detected using
reflected polarized light and grain numbers, densities,
nd cell areas were determined with an Analytic Imaging

- 38 -

Concepts image analyzer system. Due to the low
background level, the criteria for defining a cell
"labeled" was based on the presence of more than 5
clustered grains. The GAD labeled cells were found
scattered throughout the brain, enabling the
measurement of the number of grains over individual
cells. The boundary of the cell and the area covered
by a grain allowed the calculation of the number of
grains per cell. This analysis was done at a high
magnification (800X), using both reflected polarized
light and transmitted light to simultaneously visualize
the stained cell and the superimposed grains. Numbers
are means ± S.E.M. of "n" cells.

TABLE 4

CELL TYPE	GRAINS/CELL		
	GAD_{67}mRNA	GAD_{65}mRNA	GAD_{67}:GAD_{65}
Purkinje	172 ± 34 (87)*	43 ± 2 (70)	4.0
Golgi II	96 ± 8 (80)	64 ± 9 (65)	1.5
Basket	61 ± 12 (102)	16 ± 1 (57)	3.8
Stellate	55 ± 15 (65)	18 ± 3 (37)	3.1

* ± S.E.M.(n)

        In all neuronal types GAD_{67} mRNA levels are greater.
The observations with *in-situ* hybridization are
consistent with previous findings (Nitsch,
J.Neurochem., 34:822, 1980; Denner, *et al.*,
J.Neurochem., 44:957, 1985; Itoh, *et al.*, Neurochem.
Res. 6:1283, 1981) that the ratio of PLP dependent to
independent GAD activities in the cerebellum is one of
the lowest in brain regions tested. In addition, as
shown in Table 3, the order of amounts for GAD_{67} mRNA is
Purkinje > Golgi II > Basket > Stellate cells; in
contrast, for GAD_{65} mRNA, this order is Golgi II >
Purkinje > Bask t > Stellate cells.
        The expression of GAD_{65} and GAD_{67} mRNAs thus differs
among class s of neurons. The contribution of each to

- 39 -

total GAD activity in turn affects how GABA production
is regulated. For example, the substantia nigra
contains one of the highest ratios of PLP-dependent to
PLP-independent GAD activities (Nitsch, *J. Neurochem.*,
5        $\underline{34}$:822, 1980). Increasing GABA concentration in the
substantia nigra by local injection of inhibitors of
GABA catabolism is especially effective in reducing
seizure susceptibility (Gale, *Fed. Proc.*, $\underline{44}$:2414,
1985). Experimental animals undergoing seizures
10   induced by PLP-antagonists may therefore be unable to
inhibit seizure propagation because of inhibition of
GAD_{65} particularly in nerve terminals within the
substantia nigra.

15   **F.   SUBCELLULAR LOCATION OF GAD_{65} AND GAD_{67}**

The distribution of GAD_{65} and GAD_{67} was evaluated in
the S_2 and synaptosome subcellular fractions. S_2 is a
high speed supernatant consisting of the cytosol of all
cells in the brain, while the synaptosomal fraction
20   consists primarily of nerve endings (Gray, *et al.*, *J.
Anat., Lond*, $\underline{96}$:79, 1962). For these studies, whole
rat brain fractionation was performed as described by
Booth and Clark (Booth, *et al.*, *Biochem. J.*, $\underline{176}$:365,
1978). Protein concentrations were determined by
25   Schaffner and Weissman (Schaffner, *et al.*, *Anal.
.Biochem.* $\underline{56}$:502, 1973). Samples were prepared as
described (Kaiser, *et al.*, *DNA Cloning, Vol. I, A
Practical Approach*, D.M. Glover ed. (IRL Press, Oxford,
1985, pp. 38-40)', and immunoblotting was done as
30   described above using GAD-6 monoclonal antibody and K-2
antiserum. Equal amounts of protein (16µg) were added
to each lane. Autoradiography showed a linear response
of increasing amount of ^{125}I-protein A bound to antibody
with protein concentrations of 1, 3·, 10, 30, 100µgs
35   with both K-2 antiserum and GAD-6 monoclonal antibody
(data not shown).

- 40 -

The results showed that GAD_{67} was present in equal amounts in both fractions. Since the S_2 fraction contains the cytosolic proteins of glial (as well as other non-neuronal) and neuronal cells, the

5   concentration of GAD_{67} must be greater in neuronal cell bodies than in nerve endings. In contrast, the concentration of GAD_{65} was greater in synaptosomes than in S_2. These subcellular fractionation experiments suggest that, in contrast to GAD_{65}, a much greater

10   fraction of GAD_{67} is present in cell bodies of neurons than in nerve terminals. Thus, subcellular fractionation, like immunohistochemistry, shows that GAD_{65} and GAD_{67} have different subcellular distributions.

In vivo experiments utilizing inhibitors of GABA

15   synthesis and degradation have suggested that the GABA pool in neuronal cell bodies is different from that in the nerve terminals (Iadarola, *et al.*, *Mol. Cell. Biochem.*, 39:305, 1981). GABA produced by GAD_{67} may be involved more in cellular metabolism (for example, in

20   the GABA shunt) and in dendrodendritic synapses. The dendrites of granule cells in the olfactory bulb; which form dendrodendritic synapses with mitral dendrites (Shepard, *Physiol. Rev.*, 52:864, 1972) and probably release GABA (McLennan, *Brain Res.*, 29:177-184, 1971),

25   label intensely with K-2 antiserum. While not shown here, it has also been found greater GAD_{67} than GAD_{65} mRNA levels (2-3 fold) in the olfactory bulb. This distribution is consistent with the reported finding that most GAD activity in the olfactory bulb is present

30   in S_2 and P_1 (crude nuclear pellet) and not in synaptosomes (Quinn, *et al.*, *J. Neurochem.*, 35:583, 1980).

The differing subcellular distributions of GAD_{65} and GAD_{67} could result from cytoskeletal anchoring or from

35   some unknown protein targeting mechanism. Some cyt skel tal proteins have distributions that resemble GAD_{65} and GAD_{67}. For example, in cultured sympathetic

- 41 -

neurons Peng, et al. (*J Cell. Biol.*, <u>102</u>:252, 1986),
demonstrate that 84% of tau is in axons while 100% of
MAP-2 is in cell bodies and dendrites. In addition,
43kd protein, a cytoskeletal protein, is thought to
5   anchor the acetylcholine receptor to the underlying
membrane cytoskeleton (Flucher, et al., *Neuron*, 3:163,
1989).

EXAMPLE 3

10   **DETECTION OF GAD AUTOANTIBODIES IN CLINICAL SPECIMENS**
A. MATERIALS AND METHODS
1. Patient Specimens. Sera from four groups of
individuals were selected from a previous study by
Atkinson and co-workers (Atkinson, et al., *Lancet*,
15   <u>335</u>:1357-1360, 1990). These groups consisted of:
Group (1), 1 new onset IDD patients diagnosed according
to the established National Diabetes Data Group (NDDG)
criteria (Gleichman, et al., *Diabetes*, <u>36</u>:578-584,
1987) that had been referred to the University of
20   Florida, Diabetes Clinics; Group (2), 5 randomly
selected islet cell cytoplasmic antibody (ICA) negative
non-diabetic controls without any known family history
of autoimmune disease; Group (3), 13 individuals whose
sera had been collected 3 to 66 months prior to their
25   documented clinical onsets of IDD; Group (4), non-
diabetic controls and relatives, and those who were
studied prior to their onsets of IDD; and Group (5), 3
patients at risk for IDDM, but where onset has not yet
occurred. This latter group had been ascertained
30   through ongoing prospective ICA screening studies of
more than 5000 first degree relative of IDD probands,
and 8200 individuals from the general population (of
which 4813 were school children).

- 42 -

2. **Islet Cell Autoantibodi s.** ICA were assayed by indirect immunofluorescence on blood group O cryocut pancreatic (Atkinson, et al., Lancet, 335:1357-1360, 1990). All results were interpreted on coded samples, with control negative and positive sera in each batch. The degrees of ICA positivity were analyzed with the guidelines established by the Immunology Diabetes Workshop (IDW) for the standardization of ICA (Gleichman, et al., Diabetes, 36:578-584, 1987). All positive sera were titered by end point dilution, and the Juvenile Diabetes Foundation (JDF) units were determined by reference to a standard serum previously calibrated to the international JDF standard of 80 units. In the studies reported here, a positive ICA result was defined by replicate titers of 10 JDF units or greater.

3. **HLA DR Typing.** HLA DR typing was performed as adapted from the method described by Van Rood and Van Leuwen (Nature, 262:795-797, 1976), using DR trays (One Lamda Laboratories, Los Angeles, CA).

4. **Human Islet Cells.** Human pancreatic islets were isolated from cadaveric pancreases and maintained in vitro as previously described (Ricordi, et al., Diabetes, 37:413-420, 1988). The islet cells were metabolically labeled with ^{35}S methionine (Amersham, Arlington Heights, IL) in vitro (95% air/5%CO_2).

5. **Islet Cell Extractions and Immunoprecipitations.** Islet cells were extracted as previously described by Atkinson, et al. (Lancet, 335:1357-1360, 1990) with the following modifications. For immunoprecipitation studies, the islet cell lysates were precleared twice by incubation (2h, 4°C) with either control, IDD serum (100μl), or GAD-6 (Chang, et al., J.Neuro, 8:2123-2130, 1988) (1μl in 99μl of Tris buffer (Atkinson, et al., Lanc t, 335:1357-1360, 1990) for very 1000 islets. Immun complex s were then absorbed (1h 4°C) with an

- 43 -

excess of protein A Sepharose CL-4B (Pharmacia, NJ).
Aliquot volumes representing 1000 islet cells
containing unbound (precleared) lysate were then
incubated (12h, 4°C) with either IDD or control sera
5   (25µl), or GAD-6 (Chang, et al., J.Neuro, 8:2123-2130,
1988) (1µl in 25µl Tris buffer). Following another
incubation with protein A Sepharose CL-4B (1h, 4°C),
the complexes were then washed 5 times with 50mM Tris
HCL (pH 7.4) with 0.1% SDS, 1.0% Triton X-114, and 2mM
10   EDTA, and then washed again one time in double
distilled water. The protein A Sepharose CL-4B was
then boiled in Laemmli sample buffer (Laemmli, Nature,
227:680-685, 1970), and the samples were subjected to
SDS-PAGE and fluororadiography (Kodak, X-omat AR5)
15   using Enhance (New England Nuclear). Alternatively,
the autoradiographs were analyzed by a BETAGEN (Boston,
MA) analyzer. Both 64KA positive and negative sera
were used in each assay, to serve as interassay
controls. All fluororadiographs were analyzed and
20   rated as positive or negative after comparison with the
known interassay controls. Positive serum samples were
designated as 1 when a sample resulted in
immunoprecipitation of a low intensity 64,000 M_r band, 2
if a moderate intensity band was observed and 3 if the
25   intensity of the immunoprecipitated protein was high.
A similar rating procedure was employed for the
intensity of bands corresponding to immunoprecipitated
^{35}S-GAD$_{65}$ and ^{35}S-GAD$_{67}$.

6.   **Immunoprecipitations.** Immunoprecipitation of
30   bacterial lysates containing ^{35}S-GAD$_{65}$ or ^{35}S-GAD$_{67}$, and
GAD from human brain homogenate, was completed as
described above in immunoprecipitation studies of human
islet cell extractions.

7.   **GAD Assays.** Human brain homogenat s were incubated
35   with patient sera as described above in human islet
cells. Aft r absorption and washes, the protein A
agarose slurry was aliquoted into three equal volumes

- 44 -

and GAD activity was measured as described (Krieger, et al., Neurochem. $\underline{33}$:299, 1984). Briefly, Protein A agarose beads were incubated with (1-^{14}C)-glutamate (Amersham) in a designated incubation mixture (Krieger,

5   et al., J. Neurochem. $\underline{33}$:299, 1984) and production of $^{14}CO_2$ was quantitated by a liquid scintillation counter.

8. **Production of ^{35}S-GAD$_{65}$ and ^{35}S-GAD$_{67}$.** Rat GAD$_{65}$ and GAD$_{67}$ cDNAs were subcloned into a bacterial expression system as previously described. Labeling of ^{35}S-GADs

10   was completed by pulsing IPTG induced bacterium (growing in Minimal Media) for 15 minutes with TRAN ^{35}S-label (ICN). Cultures were then spun down and resuspended and sonicated in 1ml of homogenizing buffer (1mM phenylmethylsulfonyl fluoride (PMSF), 1mM 2-

15   aminoethylisothiouronium Bromide (AET) and 60mM potassium phosphate, pH 7.1). After sonication, cell debris was removed by centrifugation and protein concentration was measured (Bradford, Anal.Biochem., $\underline{72}$:248, 1986) in the supernatant (supernatant was

20   stored in aliquots at -70°C).

B.   **IMMUNOREACTIVITY OF IDDM SPECIMENS**
     Sera from patients with IDDM were tested for the ability to precipitate GAD from human brain

25   homogenates.

TABLE 5

SERA FROM IDDM PATIENTS IMMUNOPRECIPITATE GAD ACTIVITY

Patient	IDDM	Pre-IDDM Period[1]	64K[2]	JDF[3]	GAD Activity[4] cpm's
DA	+[5]	>24	3	164	13,762
DC	+	>1	3	20	1,719
RS	+	5	3	40	588
NL	+	0	2	80	440
DM	+	>1	2	10	184
C	-	na	0	0	280
C	-	na	0	0	285
C	-	na	0	0	325
C	-	na	0	0	275
C	-	na	0	0	270

[1] Expressed as months
[2] 64K titers as described in Experimental Methods
[3] The islet cell antibody test as expressed in Juvenile Diabetes Foundation (JDF) units
[4] Not adjusted for background
[5] At risk for diabetes (also, failed glucose test)
na - Not applicable

- 46 -

As shown in Table 5, the sera of four (out of five) at risk for IDDM or IDDM patients bound significantly greater amounts of enzymatically active GAD of human brain extracts than sera from control patients. In addition, sera from one of the patients was drawn in a pre-IDDM period, thus autoantibodies to GAD are present prior to the onset of IDDM symptoms (see C below).

Further experiments (results not presented) showed that the sera of two at risk IDDM patients (DA, DC) immunoprecipitated recombinantly produced ^{35}S-GAD_{65} whereas recombinantly produced ^{35}S-GAD_{67} was only recognized by sera of patient DA (and to a lesser degree than ^{35}S-GAD_{65}).

Additional studies using patient DA sera showed the presence of antibodies which recognize specific polypeptides produced in human pancreatic islet cells. Electrophoretic analysis of the bound polypeptides demonstrated the presence of autoantibodies to a 64kD component, as previously shown by others in human IDDM (Baekkeskov, et al., Nature, 298:167-169, 1982) and in animal models (Baekkeskov, et al., Science, 224:1348-1350, 1984; Atkinson, et al., Diabetes, 37:1587-1590, 1988). Prior absorption of these sera with GAD-6 monoclonal, which recognized GAD_{65} but not GAD_{67}, or with bacterially produced GAD_{65}, abolished the ability of the sera to recognize the 64kD pancreatic polypeptide. The epitopes recognized by autoantibodies to the 64kD autoantigen are thus present in GAD_{65}, indicating that the 64kD autoantigen is indeed GAD_{65}. In order to investigate the predictive value of GAD_{65}, sera drawn from patients prior to onset of clinical manifestation of IDDM were tested for autoantibodies to GAD_{65}.

TABLE 6

IDDM PATIENTS ANALYZED FOR AUTOANTIBODIES PRIOR TO THE ONSET OF DISEASE

Patient	Sex	HLA	Age Onset[1]	Pre-IDD Period[2]	JDF	64KD[3]	GAD[3]$_{65}$	GAD[3]$_{67}$
TA	M	3,2	17	11	20	2	0	1
CA	F	4,5	38	4	0	1	1	0
RA	M	2,1	5	34	0	2	1	0
TB	M	2,4	11	66	40	1	1	0
AB	M	N.D.	23	6	160	3	3	2
VC	F	4,6	15	3	40	1	0	1
JD	M	6,1	34	25	10	3	1	1
DR	F	3,4	14	42	320	2	1	0
JG	M	3,3	12	8	40	1	0	0
BR	M	3,3	5	9	0	0	1	1
KR	F	4,X	34	14	10	3	2	0
JT	F	4,6	7	10	N.D.	1	1	1

[1] Age of IDDM onset expressed as months

[2] The time interval between sera drawn and IDDM onset expressed as months

[3] 1 = lowest; 2 = medium; and 3 = highest band intensities

N.D. - not determined

- 48 -

As shown in Table 6, 9 out of 12 specimens (75%)
were immunoreactive with ^{35}S-GAD$_{65}$. In addition, two
patients (JA and VC) were immunoreactive to GAD$_{67}$, but
not GAD$_{65}$ under these conditions. Therefore, in
5   combination, autoantibodies to GAD$_{65}$ and GAD$_{67}$ were
present in 11 out of 12 (91%) of these patients sera.
This finding suggests that although autoantibodies to
GAD$_{65}$ are more common than autoantibodies to GAD$_{67}$, the
use of both recombinant GADs (GAD$_{65}$ and GAD$_{67}$) in an
10   assay would allow for greater predictability of IDDM.
Previous tests of these sera (Atkinson, et al., Langet,
335:1357-1360, 1990) demonstrated that 11 out of 12, or
92%, immunoreacted with the ^{35}S-64kD molecule from human
pancreatic islet cells. The serum which contained
15   detectable autoantibodies to the 64kD molecule and not
GAD$_{65}$ was a serum which contained the lowest titer
(or "1") for the 64kD molecule. Thus, the false
negative obtained was due to a lack of sensitivity in
this assay. Furthermore, this assay predicted IDDM in
20   one patient (BR) who was negative for 64K.
These results show that the 64kD molecule
identified in β-cells of human pancreas is identical in
size and antigenicity to rat GAD$_{65}$. Furthermore, sera
drawn from patients prior to IDDM onset contain
25   autoantibodies to GAD$_{65}$. Consequently, the GAD$_{65}$
recombinant molecule is of great utility as a
diagnostic tool for predicting IDDM. The ability of a
physician to diagnose IDDM prior to actual symptoms may
result in a greater extension of time before insulin
30   therapy is needed. The sensitivity of such
immunoassays will improve with the use of a recombinant
GAD$_{65}$ of human origin which represents the GAD form
present in β-cells of the pancreas.

35

- 49 -

EXAMPLE 4

IMMUNE PROLIFERATIVE RESPONSE TO POLYPEPTIDE

Polypeptides were synthesized using an automatic instrument (Applied Biosystems) and standard

5   conditions. These polypeptides were then tested to compare their relative ability to stimulate proliferation of splenic lymphocytes and islet infiltrating T lymphocytes (IITLs). In this study, polypeptides derived from the GAD_{65} core sequence and

10   from the homologous region of polio virus were compared. Appropriate cells were cultured for 5 days with the respective polypeptide in the presence of 5 X 10^4 irradiated spleen cells. ^3H-thymidine was added during the last 16 hours of culture.

15

TABLE 7

ANTIGEN	AMINO ACID SEQUENCE	^{3}H-THYMIDINE INCORPORATION (cpm) BY LYMPHOID CELL POPULATION	
		IITLs[a]	SPLEEN[b]
None	---	1,100	6,500
Poliovirus	MKSMCPQAQLKVKYL	900	22,500
GAD$_{65}$	ARFKMFPEVKEKGMAA	9,500	23,300

[a] Islet infiltrating T lymphocytes (3 X 10^4 cells/well)

[b] 1 X 10^5 cells/well

In these studies, there was no significant
difference in the proliferative activity of cultures of
spleen lymphocytes exposed to either the polio or the
GAD$_{65}$ polypeptides. However, both polypeptides
stimulated a T cell response which was higher than that
found in the media control. The lack of difference in
proliferation in the spleen cell population may be due
to a lower frequency of GAD polypeptide specific T
cells.

The IITL population, when evaluated in the same
manner, showed a marked difference in cell
proliferation. In this system, the response to the
GAD$_{65}$ polypeptide was 9-fold greater than that of either
the culture media or the polio polypeptide. This data
strongly suggests that the GAD$_{65}$ is an important antigen
for T cell responses in the IITL population. This data
suggests that molecular mimicry plays a role in the
pathogenesis of diabetes.

EXAMPLE 5

GAD INDUCES PROLIFERATION OF SPLEEN CELLS OF NOD MICE

Proliferative T-cell responses to β-cell antigens
(βCA) develop spontaneously in the nonobese diabetic
(NOD) mouse model in a defined chronological order.
The NOD mouse experimental model is considered the most
analogous *in vivo* system available for studying IDDM in
humans. This example describes studies on the antigen-
induced blastogenesis of spleen cells from newborn to 5
month old female NOD mice when exposed to GAD and other
peptides.

The βCAs tested included one of the two forms of
GAD (Kaufman, *et al., Science*, 232:1138-1140, 1986;
Erlander, *et al., Neuron*, 7:91-100, 1991; Kaufman, *et
al., Trends in Pharm. Sci.* (in press)), (GAD$_{65}$,
previously known as the 64K autoantigen (Baekkeskov, *et
al., Nature*, 298: 167-169, 1981; Baekkeskov, *et al.,
Nature*, 347:151-156, 1990), carboxypeptidase H (CPH)

- 52 -

(Castano, *et al.*, *J. Clin. Endoctrinol. Metab.*,
73:1197-1201, 1991), insulin (Palmer, Predicting IDDM,
Diabetes Reviews, 1:104-115, 1993) and a peptide of hsp
which has been shown to be the immunodominant
5   determinant recognized by NOD T-cells (Elias, *Proc.
Natl. Acad. Sci.*, 88:3088-3091, 1991). GAD in
particular, is a good candidate for the initial target
antigen in IDDM since autoantibodies to GAD arise early
in the natural history of the disease (Baekkeskov,
10   *supra*; Atkinson, *et al.*, *Lancet*, 335:1357-1360, 1990;
Kaufman, *et al.*, *J. Clin. Invest.*, 89:283-292,1992).
Furthermore, unlike the ubiquitous hsp, GAD is
expressed primarily in β-cells and the immunologically
privileged central nervous system (CNS) and gonads. As
15   control antigens, irrelevant prototype foreign and self
antigens including hen eggwhite lysozyme (HEL), human
serum albumin (HSA), *E. coli.* β-galactosidase (β-gal)
and murine myelin basic protein (MBP) were used.

NOD (Taconic farms) and BALB/c mice (Jackson
20   Laboratories) were kept under specific pathogen free
conditions. The mice were sacrificed at the ages
indicated and the spleen cells were tested directly *ex
vivo* for their proliferative recall response to
antigen. Single cell suspensions of spleen cells were
25   plated at 1×10^6 cells per well in 96 well microtiter
plates in 200 μl serum free HL-1 medium (Ventrex) that
was supplemented with 2mM glutamine with or without 10
μg/ml antigen (or 7 μM peptide) in triplicate cultures.
During the last 16h of the 72h culture period, 1
30   μCi[^{3}H]-thymidine was added per well. Incorporation of
label was measured by liquid scintillation counting.

Both human GAD$_{65}$ (Bu, *et al.*, *Proc. Natl. Acad.
Sci.*, 89:2115-2119, 1992) and *E. coli* β-gal (control)
were purified from recombinant bacteria on the basis of
35   a hexahistidine tag which allows their rapid affinity
purification by metal affinity chromatography (Hochuli,
et al., *Bio/T chnology*, 6:1321-1325, 1988). Bovine CPH

- 53 -

was the generous gift of L. Fricker (Albert Einstein
Col. Med.) and human insulin was purchased from Eli
Lilly.

As illustrated in Figure 8, while proliferative T-
5   cell responses were not detected at any time point to
the control antigens, a response to GAD arose at 4
weeks of age in NOD mice, concurrent with the onset of
insulitis in the colony. The blastogenesis induced by
GAD increased during the next four weeks and then
10   declined to background levels by week 16. At 6 weeks
of age, near the peak of anti-GAD reactivity, T-cell
responses to hsp appeared and increased until week 15
and then diminished as well (Figure 8). In all NOD
mice tested, hsp reactivity was preceded by an anti-GAD
15   response, suggesting that the former reactivity
developed as a secondary event during the autoimmune
process. Similarly, while no response was detected to
CPH at 4 weeks of age, a strong anti-CPH response was
observed by week 8. In some mice, a weak response to
20   insulin was observed at 12 weeks, which became more
prevalent at 15 weeks of age (Figure 8 and Table 8).
None of the antigens induced proliferation in T-cells
from age-matched control BALB/c or (NOD x BALB/c) F_1
mice, both of which do not develop insulitis or IDDM.
25   T-cell reactivity subsequently arises to other βCAs,
consistent with the inter-molecular diversification of
the autoimmune response. Thus, the autoimmune response
to GAD was the first to occur among the autoantigens
tested. In view of this, tolerization to GAD should
30   prevent the spread of autoimmunity to other βCAs and
insulitis. If this were not the case then tolerization
to GAD should have no effect on the response to these
other antigens.

Blastog n sis pr vid s an approximation of th
35   relativ clonal sizes of antigen-sp cific CD4+ T-cells
(Corradin, t al., J. Immunol., 119:1048-1053, 1977).
The data in Figure 8 shows that GAD reactive T-cells

- 54 -

"spontaneously" undergo clonal expansion concurrent with the onset of insulitis. These findings ar consistent with an endogenous priming event.

EXAMPLE 6

INDUCTION OF TOLERANCE WITH GAD

5   This example describes a study which shows that induced tolerance to GAD can ameliorate IDDM.

1.   In these experiments female NOD mice were intravenously injected at 3 weeks of age with 50 μg

10   GAD, β-galactosidase, mycobacterial hsp65 (m-hsp) or 0.1 μg of the immunodominant hsp peptide (hsp-p), in PBS. At 12 weeks of age, mice were examined for insulitis and autoantigen reactive T-cells. At this age both indications are established in untreated NOD mice.

15   Pancreatic tissue sections were stained by immunoperoxidase techniques for insulin and were counterstained with hematoxylin. Insulitis was scored in a blinded manner by examining 54 to 87 islets on 5 interrupted tissue sections from each pancreas.

20   Proliferative splenic T-cell responses induced by various antigens were performed as described above in Example 4. Data in Table 8 are expressed as the average [^{3}H]-thymidine label (cpm) incorporated in triplicate cultures.

25

TABLE 8A

GAD Induced Tolerance

Treatment	Insulitis Score[a]	N	Spleen Cell Proliferation (SI ± SEM)[b]						
			β-Gel	GAD	GAD Peptides			hsp Peptide	CPH
					#17	#34	#35		
Uninjected	2.4±0.2	5	1.0±0.2	9.5±2.1	4.8±0.4	8.0±0.1	2.9±0.2	8.7±1.0	ND[c]
β-Gal.	2.6±0.6	5	1.1±0.1	15.4±1.8	5.1±0.6	5.1±0.6	4.0±0.2	8.6±0.5	11.5±0.9
GAD 1.1±0.02	0.1±0.1	8	1.1±0.03	1.6±0.3	1.0±0.05	1.2±0.1	1.0±0.1	1.2±0.1	
hsp-p	1.7±0.4	5	1.1±0.05	5.8±0.2	4.5±0.1	4.1±0.3	4.2±0.1	1.1±0.04	4.4±0.2
m-hsp	1.8±0.5	5	1.0±0.1	4.2±0.1	3.9±0.1	3.9±0.1	3.4±0.2	1.0±0.03	4.3±0.2

* Severity of mononuclear cell infiltration was defined histologically (0 = no lymphocytic infiltration; 1 = <25%; 2 = 25-50%; 3 = 50-75%; 4 = >75%) (Qin, et al., Immunol., 150:2072-2080, 1993). Score is mean ± SE.

b Significant responses noted by solid underline, borderline responses noted by double underline.

c Not determined.

TABLE 8B

GAD Induced Tolerance

Treatment	Insulitis Score[a]	N	Spleen Cell Proliferation (SI±SEM)[b]							
			β-Gal	GAD	GAD Peptides			hsp Peptide	CPH
					#17	#34	#35		
11 peptide	≈2.5		1.0±0.1	21.1±2.2	13.7±1.5	11.4±1.5	11.3±0.7	13.3±0.9	ND
34/35 peptides (+IFA)	0.7±0.4		1.0±0.2	1.9±1.1	2.2±1.2	1.1±0.3	1.0±0.1	1.8±1.1	ND
IFA alone (+IFA)	≈2.5		1.0±0.1	8.1±0.5	5.0±0.5	4.8±0.5	4.8±0.4	8.6±0.6	ND

[a] Severity of mononuclear cell infiltration was defined histologically (0 = no lymphocytic infiltration; 1 = <25%; 2 = 25-50%; 3 = 50-75%; 4 = >75%) (Qin, et al., Immunol., 150:2072-2080, 1993). Score is mean ± SE.

[b] Significant responses noted by solid underline, borderline responses noted by double underline.

[c] Not determined.

TABLE 8C

GAD Induced Tolerance

Treatment	Insulitis Score[a] N	Spleen Cell Proliferation (SI ± SEM)[b]							
		β-Gal	GAD	GAD Peptides				hsp Peptide	CPH
				#17	#34	#35			
11 peptide	~(7)	1.4 ± 0.4	<u>12.1 ± 3.5</u>	<u>6.9 ± 0.4</u>	<u>6.4 ± 1.0</u>	<u>6.8 ± 0.3</u>	<u>8.7 ± 0.9</u>	ND
HEL peptide	(7)	1.2 ± 0.2	<u>10.7 ± 3.5</u>	<u>6.4 ± 1.4</u>	<u>5.9 ± 1.6</u>	<u>6.9 ± 1.9</u>	<u>8.0 ± 1.8</u>	ND
34/35 peptides (+ IFA)	(7)	1.0 ± 0.1	<u>4.4 ± 2.6</u>	1.8 ± 1.1	1.0 ± 0.1	1.1 ± 0.2	2.7 ± 1.3	ND

[a] Severity of mononuclear cell infiltration was defined histologically (0 = no lymphocytic infiltration; 1 = (25%; 2 = 25-50%; 3 = 50-75%; 4 =)75%) (Qin, et al., Immunol., 150:2072-2080, 1993). Score is mean ± SE.

[b] Significant responses noted by solid underline, borderline responses noted by double underline.

[c] Not determined.

- 58 -

Seventy five percent of the GAD treated mice, but none
of the controls, showed no T-cell reactivity to GAD
(indicating complete tolerization) or to other βCAs.
These mice were also completely free of insulitis

5   (score 0.0). If there were another effector T cell
population in the islets, specific for an unknown βCA,
that preceded the anti-GAD response, the release of
cytokines by this population should have promoted T-
cell responses to βCAs and insulitis (Sarvetnick, et

10   al., Nature, 346:844, 1990; Heath, et al., Nature,
359:547, 1992). Twenty five percent of the GAD-treated
mice were not completely tolerized to GAD, as evidenced
by a weak residual GAD reactivity (SI of about 3) and
displayed very limited peri-insulitis. In contrast,

15   while tolerization to both of the hsp antigens was
complete, these treatments reduced, but did not
prevent, the development of T cell responses to other
βCAs or insulitis. Thus, while the inactivation of
GAD-reactive T cells prevented β cell autoimmunity, hsp

20   tolerization only partially reduced it, as would be
expected if a secondary element was removed from the
amplifactory cascade.

In ongoing experiments examining the effects of GAD
tolerization on diabetes incidence, all of the GAD

25   treated mice (n=17, presently 37 weeks old) have normal
glucose levels, while 70% of the mice receiving control
antigens developed hyperglycemia by 19 weeks of age
(n=20). Five GAD treated mice were sacrificed at 30
weeks of age. All were free of detectable βCA reactive

30   T cells. Of these five animals, four mice were
completely free of insulitis and one mouse displayed
very limited peri-insulitis. These data show that
inactivation of GAD reactive T-cells prevents the long
term development of insulitis and diabetes.

35   2.   In a sec nd set of experiments neonatal female
NOD mice were injected intraperitoneally IFA with
peptide 11, a mixture of peptides 34 and 35 plus IFA,

- 59 -

or with IFA alone and at 12 weeks of age the mice were
examined for insulitis and autoantigen reactive T-cells
as in Example 6.1. Proliferative splenic T-cell
responses induced by the various antigens were
5   performed as in Example 4, and data in Table 8B are
expressed as the average [^{3}H]-thymidine label (cpm)
incorporated in triplicate cultures.

The data in Table 8B show that tolerization with
control peptide 11 did not prevent auto antibody
10   response to GAD or to GAD peptides 17, 34 or 35. Nor
was response to hsp peptide prevented by tolerization
with peptide 11. IFA alone was somewhat effective at
suppressing immune response. By contrast, tolerization
with the mixture of peptides 34 and 35 suppressed the
15   autoimmune response of spleen cell proliferation to all
species tested: β-gal, GAD, GAD peptides 17, 34 and
35, and hsp peptide. In addition, tolerization to GAD
peptides 34 and 35 greatly reduces insulitis but does
not completely prevent it as whole GAD65 does.
20   3.   In a third set of experiments female NOD mice
were intravenously injected at three weeks of age with
peptide 11 (control), a mixture of peptides 34 and 35
plus IFA, or with HEL peptides and at 12 weeks of age
the mice were examined for insulitis and autoantigen
25   reactive T-cells as in Example 6.1. Proliferative
splenic T-cell responses induced by the various
antigens were performed as in Example 4, and data in
Table 8C are expressed as the average [^{3}H]-thymidine
label (cpm) incorporated in triplicate cultures.

30   The data in Table 8C show that tolerization with
control peptide 11 did not prevent auto antibody
response to GAD, to GAD peptides 17, 34 or 35 or to hsp
although the response was not as great as in Example
6.2. Nor was response to hsp peptid pr vented by
35   immunization with peptide 11. By contrast,
immunization with the mixture of peptides 34 and 35
plus IFA suppressed the autoimmune response of spleen

- 60 -

cell proliferation to all species tested: β-gal, GAD, GAD peptides 17, 34 and 35, and hsp peptid .

EXAMPLE 7

CHARACTERIZATION OF GAD-REACTIVE T-CELLS

5   This example describes studies on GAD-Reactive T-Cells for additional properties that distinguish activated/memory from resting/naive lymphocytes.

In one series of experiments γ interferon (IFNγ) was measured by ELISA in culture supernatants (CSN) of

10   spleen cells of 6-9 week old mice after challenge with GAD or control antigens HEL and MBP. Additionally, the frequency of antigen specific, IFNγ-producing cells was determined by an ELISA spot technique (T. Taguchi, et al., J. Immunol., 145:68-77, 1990). Frequency of

15   antigen-induced, spot forming cells (SFC) among 10^3 spleen cells is represented in Figure 9(a). Values are the mean + SEM from 5 individual female NOD mice, each tested in triplicate cultures with or without antigen. Results from a single experiment are shown. These are

20   representative of 3 separate experiments.

In performing these experiments, freshly isolated spleen cells were cultured with or without antigen as described in Example 4. CSN were taken after 48 h and the concentration of IFNγ was determined by ELISA

25   (Macy, et al., FASEB J., 3003-3009, 1988). IFNγ specific monoclonal antibody (mAb) R4-6A2 (Pharmingen) was used as the capturing reagent and biotinylated mAb XMG 1.2 (Pharmingen, also specific for IFNγ) was used in conjunction with streptavidin-alkaline phosphatase

30   (Zymed) and p-nitrophenol for detection of bound lymphokine. Recombinant murine IFNγ (Pharmingen) was used as a standard. ELISA spot assays for the detection of antigen-specific, IFNγ-producing cells w re performed as described (Taguchi, et al., J.

35   Immunol., 145:68-77, 1990). After a 24h pre-activation culture of spleen cells with our without antigen, cells were transferred by serial dilution to 96 well

- 61 -

microtiter plates (Millipore) that had been pre-coated
with mAb R4-6A2. After 24h, the cells were removed and
IFNγ spots were visualized using XMG 1.2-biotin in
conjunction with nitroblue terazolium-

5   bromochloroindolyl phosphate substrate (Sigma). Spots
were counted visually and the frequency of antigen
specific cells was determined from the difference
between the number of spots seen with and without
antigen.

10   As shown in Figure 9(a), when freshly isolated T-
cells from 6-9 week old NOD mice were challenged with
GAD or control antigens, high concentrations of IFNγ
were detected only in cultures containing GAD,
suggesting that the GAD specific T-cells had been pre-

15   activated *in vivo*, since only pre-activated T-cells
(Th1) produce IFNγ within 48 hours after antigen
recognition (Ehlers, *et al.*, *J. Exp. Med.*, 173:25-36
1991; Croft, *et al.*, *J. Exp. Med.*, 176:1431-1437,
1992). In contrast, T-cells from age matched BALB/c

20   mice did not respond to GAD or to control antigens by
IFNγ production (data not shown).

        Results of the ELISA spot assay to measure directly
the frequency of GAD-specific T-cells showed that while
in 6-9 week old NOD mice, T-cells reactive to control

25   antigens constituted approximately 1 in 10^5 cells in the
spleen, the frequency of GAD-reactive T-cells was about
two orders of magnitude higher, ranging from 90-291
cells per 10^5 cells (Figure 9(a), confirming the data
obtained by proliferation assays (Figure 8) that these

30   cells had been clonally expanded *in vivo*.

        In another series of experiments, GAD specific T-
cells were characterized for expression of the cell
surface marker L-selectin, since murine T-cells convert
from an L-selectin[+] (L-sel[+]) to an L-selectin[-] (L-sel[-])

35   phenotype upon activation (Bradley, *et al.*, *J.
Immunol.*, 148:324-331, 1992).

- 62 -

To perform these studies, pooled spleen cells from 3 to 4 ag matched mice were panned on plates coated with goat-anti-mouse Ig (Zymed) to remove adherent macrophages as well as B cells. Next, CD8+ cells were coated with mAb 58.6-72 (ATCC) and removed by panning over plates coated with goat-anti-rat Ig (Zymed). The non-adherent CD4+ cell fraction was labeled with anti-L-selectin mAb MEL-14 (ATCC) and panned on goat-anti-rat Ig coated plates. Both the adherent (CD4+ L-sel$^+$) and non-adherent (CD4+,L-sel$^-$) fractions were sampled. Purity of the cell fraction was assessed by FACS analysis; cells were $>$90% CD4+ and $>$95% enriched for the L-sel$^-$ or L-sel$^+$ phenotype. The purified cell fractions were tested for GAD reactivity by seeding them at 2 x 10^5 cells per well in 96 well microtiter plates with or without antigen. Irradiated (3000 rad), unseparated spleen cells of 3 week old NOD mice were added at 5 x 10^5 cells per well as a source of antigen presenting cells. Supernatants of triplicate cultures were taken 48h later and their IFNγ content was determined by ELISA.

The results of this study showed that by 2-3 weeks of age, GAD reactive T-cells could not be detected in either the L-sel$^+$ or the L-sel$^-$ population, consistent with a low frequency of antigen reactive precursors at this time point. However, by 6 weeks of age high levels of IFNγ were induced by GAD (but not by control antigens) in the L-sel$^-$ (but not the L-sel$^+$) subpopulation of CD4+ cells (Figure 9(b)).

The increase in clonal size of GAD reactive T-cells, their production of IFNγ and their L-sel$^-$ phenotype provide three independent lines of evidence that a potentially pathogenic (Ando, et al. Cell Immunol., 124:132-143, 1989) Th1 type T-cell respons is sp ntan ously primed to GAD in vivo early in NOD development.

- 63 -

EXAMPLE 8

CHARACTERIZATION OF AD SPECIFIC T-CELL
DETERMINANT RECOGNITION

The fine specificity of the anti-GAD T-cell
response was mapped using a set of 38 peptides
(numbered successively from the N-terminus) that were
20-23 amino acids (aa) long and span the entire GAD_{65}
(Bu, et al., Proc. Natl. Acad. Sci., 89:2115-2119,
1992) sequence with 5aa overlaps (Figure 10).

Spleen cells were tested from 4 (Figure 10a), 5
(Figure 10b) and 7 (Figure 10c) week old NOD mice for
proliferative responses (as described in Example 4) to
the GAD peptides. Peptides were present in cultures at
7 µM and the label was added during the last 16 hours
of a 5-day culture. The peptides were synthesized
using standard Fmoc chemistry and purified by reverse
phase HPLC (Advanced Chemtech). The sequence of
stimulatory peptides are shown below in Table 9.

TABLE 9

Peptide Number	GAD Region	Amino Acid Sequence
6	78-97	KPC**S**C**S**KVD**V**NYAFLHATDL
17	247-266	NMYAM**M**IAR**F**KMFPEVKEKG
23	335-356	TAGTTVYGAFDPLLAVADICKK
32	479-498	EYLY**NI**IKNREGYEMVFDGK
34	509-528	**I**PPSLRYLEDNEERMSRLSK
35	524-543	SRLSKVAPVIKARMMEYGTT
36	539-558	EYGTTMVSYQPLGDKVNFFR
38	566-585	ATHQDIDFLIEEIERLGQDL

Murine and human GAD_{65} are 95% identical at the
amino acid level (555/585) and are 98% conserved, with
m st of the differ nces l calized near their N-termini.
The underlined amino acid in the stimulatory p ptide

- 64 -

sequ nces above are conservatively substituted in
murine GAD$_{65}$. In separat experiments, the murin f rm
of key peptides (#17 and #34) were tested and produced
similar results.

5   As shown in Figure 10, peptides that triggered
stimulation indices >3 are indicated as black bars.
These peptides did not induce proliferation in T-cells
from NOD mice <3 or >16 weeks in age, or from control
(BALB/c x NOD)F1 mice (data not shown). The data are
10   represented as the mean SI ± standard error calculated
from 3-6 individual mice tested twice in each age
group. Characteristic results for peptide induced
blastogenesis in individual mice are shown in Table 6.
The first detectable response, at 4 weeks of age, was
15   confined to the carboxy-terminal region of GAD, and
involved two adjacent peptides (aa 509-528 and 524-543,
peptides #34 and #35, respectively, Figure 10a). At 5
weeks of age, responses to an additional determinant
(aa 247-266, peptide #17, which contains a region of
20   sequence similarity with Coxsackievirus (Kaufman, et
al., J. Clin. Invest., 89:283-292, 1992) (Figure 10b)
were regularly recorded. During the next two weeks,
responses to peptide #17 (aa247-266) increased and T-
cell autoimmunity spread to two additional peptides at
25   the carboxy terminus (aa 479-498 and 539-558; peptides
#32 and #36 respectively, Figure 10c). Subsequently,
reactivity to the GAD peptides declined (data not
shown), paralleling the loss of response to the whole
protein (Figure 8). It is unclear why the initial T-
30   cell response to βCAs fades in NOD mice. Possible
explanations include: a) immune regulatory mechanisms;
b) exhaustion of the response due to the continuous
stimulation by the endogenous antigen; and c) induction
of anergy in specific T-cells owing to their
35   recognition of the autoantig n n "non pr fessi nal"
antigen presenting cells such as the β cells themselves
(Markmann, et al., Nature, 336:476-479, 1988).

- 65 -

The gradual diversification of the primed
autoreactive T-cell repertoire that was observed in
this naturally occurring autoimmune disease parallels
the shifts in T-cell recognition recently observed in
5   experimentally induced autoimmunity to the CNS where
autoreactivity spreads both intra- and intermolecularly
among CNS proteins (Lehmannn, et al., Nature, 358:155-
157, 1992; Perry, et al., J. Neuroimmunol., 33:7-15,
1991; Watanabe, et al.,Nature, 305:150-153, 1983;
10   Liebert, et al., J. Neuroimmunol.,17:103-118, 1988).
Apparently, lymphokine secretion by the first wave of
autoantigen specific T-cells in the target organ
results in up-regulation of antigen presentation and
creates a microenvironment that favors priming of
15   additional autoreactive T-cells (Lehmann, et al.,
Immunol. Today, 14:203-208, 1993; Sarvetnick, et al.,
Nature, 346:844-847, 1990; Heath, et al., Nature,
359:547-549, 1992). Since hsp reactive CD4+ T-cells
are capable of inducing IDDM (Elias, et al., Proc.
20   Natl. Acad. Sci., 87:1576-1580, 1990; Elias, et al.,
Proc. Natl. Acad. Sci., 88:3088-3091, 1991), their
recruitment into the activated T-cell pool, along with
T-cells reactive to other βCAs, probably reflects an
amplificatory cascade that eventually leads to β cell
25   destruction.

In summary, the data above establish GAD as a
critical target antigen in the pathogenesis of IDDM in
NOD mice. The results show that T-cell responses to
βCAs diversify both intramolecularly and
30   intermolecularly as the disease progresses, consistent
with a dynamic autoimmune repertoire (Lehmann, et al.,
Immunol. Today, 14:203-208, 1993). However,
interference with the early autoreactive T-cell
population can prevent the recruitment of additional
35   autoantigens into the primed repertoire thereby halting
a cascade of autoimmune respons s that eventually l ads
to β cell destruction. As a similar autoimmune

- 66 -

progression is also likely to occur during the
development of human IDDM (Palmer, J.P., Predicting
IDDM, *Diabetes Reviews*, 1:14-115, 1993; Atkinson, et
al., *Lancet*, 339:458-459, 1992), these findings suggest
5   that peptide-based immunotherapeutic agents would be
useful in predicting and ameliorating human IDDM.

EXAMPLE 9

AUTOANTIBODY REACTIVITY WITH GAD FRAGMENTS

This example describes a study which examined the
10   variability in recognition of epitopes in human GAD_{65}
polypeptides by IDDM autoantibodies in sera of human
patients.

Portions of human GAD_{65} cDNA were amplified by the
polymerase chain reaction (PCR; Saiki, et al., *Science*,
15   239:487, 1988) to produce DNA segments encoding three
polypeptide segments: amino acid residues 1-224
(segment A); 224-398 (segment B); and 398-585 (segment
C).   Each construct also contained a T_7 promoter, a
consensus sequence for the initiation of translation
20   and an initiating methionine codon (Korak, M., *J. Cell
Biol.*, 108:229, 1989).   Each PCR product was then
trascribed *in vitro* with T_7 RNA polymerase and
translated *in vitro* in a rabbit reticulocyte cell-free
system in the presence of ^{35}S-methionine, using
25   conditions recommended by the supplier (Amersham Corp.,
Arlington Heights, IL).   Each test serum (30 μl) was
incubated with the resulting ^{35}S labeled-polypeptides.
The bound peptides were isolated with PAS and analyzed
by SDS-PAGE in 12% polyacrylamide and autoradiography.

30

TABLE 10

IDDM PATIENT SERA REACTIVITY WITH GAD SEGMENTS

	PATIENT	SEGMENT		
		A	B	C
5	Control (N=7)	−	−	−
	052	−	+	+
	723	−	−	−
	705	−	+	+
10	UC2	−	+	+
	N.L.	−	−	−
	L.I.	−	−	−
	T.L.	−	−	−
	P.T.	−	+	−
15	J.D.	−	−	−
	B.Y.	−	+	+
	M.C.	−	−	−
	R.S.	−	−	−
	K.O.	−	−	−
20	T.B.	−	−	−
	S.M.	−	−	−
	A.W.	−	+	−
	J.B.	−	+	+
	J.A.	−	−	−
25	P.C.	−	+	+
	L.R.	−	−	−
	J.M.	−	+	−
	G.A.	−	−	−

- 68 -

As shown in Table 10, none of the sp cimens had
detectable levels of antibodies to the amino terminal
third (segment A) of GAD whereas 9 patients (41%) had
antibodies reactive with the middle third (segment B)
and 6 patients (27%) had antibodies to the carboxyl-
terminal third (segment C) of GAD.

EXAMPLE 10

PREDICTION OF INCIPIENT IDDM BY GAD

EPITOPE RECOGNITION PATTERN

The increasing likelihood of an IDDM interventive
therapy and the (recently acknowledged) benefits of
managed glucose homeostasis in preventing IDDM
associated complications makes the early detection of β
cell autoimmunity before clinical IDDM onset and in
NIDDM patients (10% of whom eventually convert to IDDM)
a crucial goal. Autoanitbodies to GAD may provide the
earliest and most reliable marker of impending IDDM
among the molecularly defined IDDM associated
autoantigens. To determine whether GAD peptides will
bind to IDDM associated autoantibodies the following
study was conducted.

A set of peptides (20-23 amino acids in length,
with 5 aa overlaps) that span the human GAD65 molecule
were synthesized to determine whether sera from most
individuals at risk, pre-IDDM and with IDDM (in
contrast to healthy controls) do in fact produce
antibodies that differentially recognize GAD65 linear
epitopes distributed throughout the molecule.

Patient sera and most control sera were those used
in a previous study (Kaufman, et al., J. Clin.
Investigation, supra) All samples were coded and tested
in a blind manner. Peptides were synthesized using an
automatic instrument (Applied Biosystems, Foster City,
CA) and standard conditions. Peptides were dissolved
in 60 mM sodium bicarbonate buffer (pH 9.6) at 20 ug/ml
and 100 ul of each was add d to duplicated wells of a
96 well Nunc-Immuno Plate. P ptides w r allowed t

- 69 -

bind at 4°C overnight. The plates were then washed
three times with PBS + 0.1% Tween 20 (wash buffer),
after which the plates were pre-absorbed with 3% BSA in
sodium bicarbonate buffer for 0.5 hours at 37°C, or at
5   room temperature overnight. The plates were then
washed 5 times with the above wash buffer. 100 ul of
serum at a 1/300 dilution in PBS + 0.1% Tween 20 and 1%
BSA was added to each well and antibodies were allowed
to bind for 1 hour at 37°C. The plates were washed 5
10   times with wash buffer. 100 ul of a 1/600 dilution of
HRP-goat anti-human IgG (BRL, Gaithersberg, MD) was
added to each well and allowed to bind for 1 hour at
37°C. The plates were then washed 7 times and 100 ul
of substrate buffer was added to each well for 30
15   minutes at room temperature. The color development was
measured at 410 nm using an ELISA plate reader (ICN,
Biomedicals, Costa Mesa, CA). Positive sera were
defined as: OD_{410} of the sample/negative control ≥3.0.

20   The data shown in Table 11 establish that a number of
GAD peptides were recognized by patients previously
shown to be 64K positive, but not by control sera.
Each patient showed a different pattern of GAD epitope
recognition. Peptides 20, 21 and 25, were each
25   recognized by 6/8 patients, and none of the controls--
with the exception of peptide 25 which was recognized
by 1 out of 13 controls. Based on immunoreactivity to
2 of these peptides (#20 and 21) 7/8 (88%) of the
patients (and none of the controls) could be identified
30   as possessing GAD autoantibodies. Peptides 3, 6, 22,
25 and 37 were each recognized by only 25-37% of the
patients (and none of the control sera), but taken
together, 75% of the patients recognized at least one
of thes . Peptides 5, 9 and 24 were oft n positiv for
35   immunor activity by both control and patient sera.
This level of sensitivity is comparable to the best
currently available assays using whole GAD65 purified

- 70 -

from brain or recombinant organisms. Besides avoiding
laborious antigen purification, peptide based
autoantibody screening, together with PCR based HLA
typing, may reveal epitope recognition patterns
5   associated with progression or lack of progression to
IDDM and its associated complications. Individuals
determined to be at high risk could then consider
therapeutic intervention.

It should also be noted that the GAD peptides
10   recognized by autoantibodies were different from those
recognized by NOD GAD reactive T cells in Example 6.

TABLE 11

EPITOPE RECOGNIATION OF HUMAN GAD65 PEPTIDES

No.		PEPTIDE	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38
	Controls																																							
	A.P.					x				x																													
	P.T.					x																																	
10	5748					x				b																													
	5620					x				b																	b				b								
	4380					x				b															x														
	S.M.					x				x															x			b					b						
15	FA8																					b			x														
	FA12					b																			x														
	1688					b																			x														
	FA9					x																			x														
	H.B					b																			x														
	FA2					x																			x														
20	FA11					x																			x														
	IDDM									2																1													
	At Risk																																						
25	052			b		x	x			b				b		b	b			x					x				b										b
	825					x	x				x									x					x	b			x			b							
	356					x				x												x			x	x													
	L.I.					x			x		b										b				x								b						

PEPTIDE

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38
Pre-IDDM																																						
J.A.																																						
723			X		X															X	X			X	X													
P.T. (1)			b		X				X											X	b			X	b				X			b					X	
(2)					X															X				X	X				X			b		b	X		X	
J.B. (1)			b	b	b		b	b	b	b	b								b	b				X	b				b									
(2)			b		X	b	b	b	b	b	b									b	b			X	X													
R.S. (1)			X		X				b	b	b								b					X	X													
(2)			X		X			b	b	b	b													X	X													
B.Y. (1)			X		X						X										b			X														
(2)					X																			X														
J.B. (1)			b		b		b	b	b	b	b								b	b	b			X	b				b									
(2)					X		b	b	b	b	b									b	b			X	X				b									
At Onset																																						
705					X			X												X	X	X		X	X				X							X	X	
S.H.	b		X		X															X	X	X		X	X							b		b		X		b
291			b	X	X	X	b													X	X	X		X	X		X		X			b		X				
048			X		X	X			X											X				X	X				X			X		X		b	X	X
counts			5	1		2	2	2	3	2	2								1	7	7	2			8		1		4			1		1	1	1	2	1

X = positive for immunoreactivity as defined by OD/background≥3.0

b = borderline response (OD/background =2.5-2.9).

- 73 -

EXAMPLE 11

GAD IMMUNIZATION PROTECTS NOD MICE FROM IDDM

The availability of cDNAs encoding GAD65 allows the
testing of this molecule in new interventive therapies
5   designed to interfere with GAD-specific T cells. Tests
were conducted to examine the ability of GAD65
immunization to protect NOD mice at 8 weeks of age, a
time at which T cell responses to a number of b cell
antigens and insulitis is already well established. If
10   GAD immunotherapy was effective at this stage, it would
hold promise for treatment in humans in which the
autoimmune process has already been established.

METHODS

Antigens

15   An IPTG inducible T7 expression vector was used to
express both human GAD65 and *E. coli* b galactosidase
(β-gal). In IPTG induced recombinant *E. coli*, GAD and
β-gal constitute about 10-20% of the total bacterial
protein. However, almost all of the GAD was in
20   inclusion bodies, which could be isolated and
extensively washed to obtain material that is about 80%
GAD. We then did affinity purifications of GAD and β-
gal on the basis of a hexa-histidine "tag" which was
attached to GAD during the subcloning process. These
25   extra histidine residues allow the rapid affinity
purification (Novagen) of GAD by metal affinity
chromatography (Hochuli, *et al.*, Bio Technology,
<u>6</u>:1321-1325, 1988). The inclusion body material is
solubilized in 6M guanidine hydrochloride (GHCL), 10mm
30   β-mercaptoethanol and 1% triton X-100. After binding
to the column, the column was extensively washed with
GHCL and 8M urea in phosphate buffers. Only the
central peak GAD fraction was utilized for subsequent
studies. Human GAD65 shares 96% amino acid sequence
35   identity with murine GAD65, with most of the amino acid
differences being conservative substitutions.

- 74 -

The GAD preparation appeared t be free of
immunologically detectable contaminants. It also
appeared to be free of bacterial contaminants on
overloaded silver stained gels. Analysis by a national
5   reference laboratory found ⟨0.06ng LPS/ug GAD. Human
GAD65 did not induce T cell proliferation in ⟨4 or ⟩16
week old NOD or control BALB/c or (NOD/BALB/c) F1
spleen cells. The results using synthetic GAD peptides
(Figure 10) precisely parallel the data using whole
10   recombinant GAD (Figure 8). Other antigens described
herein elsewhere that are not involved in IDDM (such as
the beta galactosidase) did not induce NOD T cell
responses. After immunizing mice with GAD, we were
unable to detect cross reactive T cell responses in
15   recall experiments with other proteins that were
purified from recombinant *E. coli* by the same metal
affinity chromatography procedure. Amino acid sequence
analysis of GAD and β-gal each gave a single expected
amino acid N-terminal sequence. If there had been
20   appreciable endotoxins, heat shock proteins, or other
contaminants present in the GAD preparation, spleen,
PBMC (Atkinson, *et al.*), and T cell proliferation
responses that were not disease specific would have
been expected.
25   Breeder mice were purchased from Taconic Farms and
housed under specific pathogen-free conditions. Only
female NOD mice were used in this study. The average
age of IDDM onset in unrelated females in the colony
was 22 weeks. Insulitis is generally observed
30   beginning at 4 weeks of age. T cell responses to GAD,
HSP, CPH were found by 6 weeks in age. The incidence
of IDDM in female mice is 70-90% by one year of age.
Immunizations

At 8 weeks of age, 25 ug GAD or control β-gal. was
35   inject d intraperitoneally (ip) in 100 ul of incomplet
Freunds adjuvant (IFA). Because there may be a
requirement for continual antigen presentation

- 75 -

(Ramsd ll, t al., Science, 257:1130-1133, 1992) mice
were treated again every 6 weeks. Urine glucose levels
were monitored twice weekly. After observing above
normal glucose in urea, blood glucose levels were
5   monitored twice weekly. Two consecutive blood glucose
level readings of 300 mg/ml was considered as IDDM
onset, after which the mice were sacrificed and spleen
cells were tested as described above in Example 6 for
evidence of spleen cell proliferation.
10   Immunization of 8 week old NOD mice produced a
clear delay in the onset of IDDM compared to control β-
gal immunized mice (Fig. 11). While two of the GAD
immunized mice (open circles) developed IDDM at about
the normal age of onset (20 weeks), the other 8 GAD
15   immunized mice showed no signs of hyperglycemia until
36 weeks in age. Four of the GAD treated mice
developed IDDM between 37 and 40 weeks in age. Four of
the GAD treated mice currently remain disease free (at
52 weeks of age). In contrast, the majority of β-gal
20   injected mice (closed circles) had hyperglycemia by 22
weeks of age and 6/10 developed IDDM by 27 weeks in
age. At 52 weeks of age, 2 of the β-gal treated mice
remain disease free. This experiment shows that GAD
immunization significantly delayed ($\langle$0.02) or prevented
25   diabetes of NOD mice in which β cell autoimmunity has
already significantly progressed.
         β cell autoimmunity is already well established at
8 weeks of age, and it is likely to also be in
individuals determined to be at risk for IDDM on the
30   basis of circulating autoantibodies. Although the
mechanism of this protection is not clear, periodic
injections of GAD have a profound moderating effect on
the induction of disease.

- 76 -

TABLE 11

AMINO ACID SEQUENCES FOR GAD65

	1	MASPGSGFWSFGSEDGSGDS
5	2	GSGDSENPGTARAWCQVAQKFTG
	3	QKFTGGIGIGNKLCALLYGD
	4	LLYGDAEKPAESGGSQPPRA
	5	QPPRAAARKAACACDQKPCSC
	6	KPCSCSKVDVNYAFLHATDL
10	7	HATDLLPACDGERPTLAFLQ
	8	LAFLQDVMNILLQYVVKSFDRS
	9	SFDRSTKVIDFHYPNELLQE
	10	ELLQEYNWELADQPQNLEEILM
	11	EEILMHCQTTLKYAIKTGHP
15	12	KYGHPRYFNQLSTGLDMVGL
	13	DMVGLAADWLTSTANTNMFT
	14	TNMFTYEIAPVFVLLEYVTL
	15	EYVTLKKMREIIGWPGGSGD
	16	GGSGDGIFSPGGAISNMYAM
20	17	NMYAMMIARFKMFPEVKEKG
	18	PEVKEKGMAALPRLIAFTSE
	19	AFTSEHSHFSLKKGAAALGI
	20	AALGIGTDSVILIKCDERGK
	21	DERGKMIPSDLERRILEAKQ
25	22	LEAKQKGFVPFLVSATAGTT
	23	TAGTTVYGAFDPLLAVADICKK
	24	DICKKYKIWMHVDAAWGGGLLMS
	25	GLLMSRKHKWKLSGVERANS
	26	ERANSVTWNPHKMMGVPLQC
30	27	VPLQCSALLVREEGLMQNCNQ
	28	QNCNQMHASYLFQQDKHYDL
	29	KHYDLSYDTGDKALQCGRHV
	30	CGRHVDVFKLWLMWRAKGTTG
	31	KGTTGFEAHVDKCLELAEYLYN
35	32	EYLYNIIKNREGYEMVFDGK
	33	VFDGKPQHTNVCFWYIPPSL
	34	IPPSLRTLEDNEERMSRLSK

- 77 -

35 SRLSKVAPVIKARMMEYGTT

36 EYGTTTMVSYQPLGDKVNFFR

37 VNFFRMVISNPAATHQDIDF

38 ATHQDIDFLIEEIERLGQDL

5

The invention now being fully described, it will be
apparent to one of ordinary skill in the art that many
changes and modifications can be made without departing
10 from the scope of the invention.

- 78 -

CLAIMS

1.   A polypeptide consisting essentially of an amino
     acid sequence selected from the group consisting
     of:
               KPCSCSKVDVNYAFLHATDL;
               NMYAMMIARFKMFPEVKEKG;
               TAGTTVYGAFDPLLAVADICKK;
               EYLYNIIKNREGYEMVFDGK;
               IPPSLRYLEDNEERMSRLSK;
               SRLSKVAPVIKARMMEYGTT;
               EYGTTMVSYQPLGDKVNFFR;
               ATHQDIDFLIEEIERLGQDL;
               LAFLQDVMNILLQYVVKSFDRS;
               EEILMHCQTTLKYAIKTGHP;
               DERGKMIPSKLERRILEAKQ;
               KHYDLSYDTGDKALQCGRHV;
               AALGIGTDSVILIKCDERGK;
               GLLMSRKHKWKLSGVERANS;
               LEAKQKGFVPFLVSATAGTT; and
               VNFFRMVISNPAATHQDIDF.

2.   An isolated polynucleotide sequence encoding a
     polypeptide of claim 1.

3.   The polynucleotide of claim 2 which is DNA.

4.   A method for detecting autoantibody to GAD in a
     patient specimen which comprises contacting the
     specimen with a GAD fragment and determining
     whether autoantibody binds to the fragment.

5.   The method of claim 4, wherein the fragment is a
     polypeptide of at least about 16 amino acids in
     length and is selected from the amino acid
     sequence from about amino acid 224 to about amino
     acid 585 of GAD$_{65}$.

6.   The method of claim 4, wherein the fragment is a
     polypeptide of claim 1.

7.   Th m thod of claim 4, wherein the specimen is
     blood.

8. The method of claim 4, wherein the peptide is detectably labeled.

9. The method of claim 8, wherein the detectable label is selected from the group consisting of a radioisotope, a fluorescent compound, a colloidal metal, a chemiluminescent compound, a bioluminescent compound, a phosphorescent compound, and an enzyme.

10. The method of claim 4, wherein the peptide is bound to a solid phase.

11. A monoclonal antibody to the peptide of claim 1.

12. A hybridoma cell line capable of producing the monoclonal antibody of claim 9.

13. A vector containing the polynucleotide of claim 2.

14. A host cell transformed with the polynucleotide of claim 2.

15. A method for ameliorating a GAD associated autoimmune disorder in a patient having or at risk of having the disorder, which comprises administering to the patient a therapeutically effective amount of whole GAD_{65} or a therapeutically effective fragment thereof.

16. The method of claim 15, wherein the therapeutically effective fragment is a polypeptide of at least about 16 amino acids in length and is selected from the amino acid sequence from about amino acid 224 to about amino acid 585 of GAD_{65}.

- 80 -

17.   The method of claim 15, wherein the therapeutically effective fragment is selected from the group consisting of:

      KPCSCSKVDVNYAFLHATDL;

      NMYAMMIARFKMFPEVKEKG;

      TAGTTVYGAFDPLLAVADICKK;

      EYLYNIIKNREGYEMVFDGK;

      IPPSLRYLEDNEERMSRLSK;

      SRLSKVAPVIKARMMEYGTT;

      EYGTTMVSYQPLGDKVNFFR; and

      ATHQDIDFLIEEIERLGQDL.

18.   The method of claim 15, wherein the therapeutically effective fragment is selected from the group consisting of:

      KPCSCSKVDVNYAFLHATDL;

      LAFLQDVMNILLQYVVKSFDRS;

      EEILMHCQTTLKYAIKTGHP;

      DERGKMIPSKLERRILEAKQ;

      KHYDLSYDTGDKALQCGRHV;

      AALGIGTDSVILIKCDERGK;

      GLLMSRKHKWKLSGVERANS;

      LEAKQKGFVPFLVSATAGTT; and

      VNFFRMVISNPAATHQDIDF.

19.   The method of claim 16, wherein the disorder is IDDM.

20.   The method of claim 16, wherein the disorder is stiff man disease.

21.   The method of claim 16, wherein the administration is enteral.

22.   The method of claim 21, wherein the enteral administration is oral.

23.   The method of claim 15 or 16, wherein the administration is parenteral.

24.   The method of claim 23, wherein the parenteral administration is by subcutan ous, intramuscular, intraperitoneal, intracavity, transdermal, intranasal, or intravenous inj ction.

- 81 -

25. The m thod of claim 15, wherein said
administration is at a dosage of about 0.01
mg/kg/dose to about 2000 mg/kg/dose.

26. The method of claim 15, wherein the polypeptide
is therapeutically labeled.

27. The method of claim 26, wherein the therapeutic
label is selected from the group consisting of a
radioisotope, a drug, a lectin, and a toxin.

28. A method for detecting the status of a GAD
associated autoimmune disorder in a patient which
comprises contacting a T-cell of the patient with
at least one polypeptide of claim 1 and detecting
the response of the T-cell to the peptide.

29. The method of claim 26, wherein the response of
the T-cell is stimulation.

30. The method of claim 27, wherein the stimulation
is measured by cellular uptake of radiolabelled
nucleoside.

31. The method of claim 30, wherein the nucleoside is
^{3}H thymidine.

32. A kit useful for the detection of antibody to the
polypeptide of claim 1 in a specimen suspected of
containing such antibody, the kit comprising
carrier means compartmentalized to receive in
close confinement therein one or more containers
comprising a container containing the polypeptide
of claim 1.

33. A kit useful for determining the status of a GAD
associated disorder in a specimen of a patient,
the kit comprising carrier means
compartmentalized to receive in close confinement
therein one or more containers comprising a
container containing a polypeptid of claim 1.

- 82 -

34. A method for detecting a GAD associated autoimmune disorder in a patient having or at risk of having the disorder, which comprises administering to the patient a diagnostically effective amount of a diagnostically effective fragment of GAD$_{65}$.

35. The method of claim 34, wherein the diagnostically effective fragment is a polypeptide of at least about 16 amino acids in length and is selected from the amino acid sequence from about amino acid 34 to 573 of GAD$_{65}$.

36. The method of claim 35, wherein the diagnostically effective fragment is selected from the group consisting of:

        KPCSCSKVDVNYAFLHATDL;

        LAFLQDVMNILLQYVVKSFDRS;

        EEILMHCQTTLKYAIKTGHP;

        DERGKMIPSKLERRILEAKQ;

        KHYDLSYDTGDKALQCGRHV;

        AALGIGTDSVILIKCDERGK;

        GLLMSRKHKWKLSGVERANS;

        LEAKQKGFVPFLVSATAGTT; and

        VNFFRMVISNPAATHQDIDF.

37. The method of claim 35, wherein the disorder is IDDM.

38. The method of claim 35, wherein the disorder is stiff man disease.

feline (from cDNA) YEIAPVFVLMEQITLKKMREIVGWSSKDGDGIFSPGGAISNMYSIMAARYKFFPEVKTKG

rat (from peptide) YEIAPVFVLLEYV-----REIIGWPGGS-DGIFSPGGAISN-YAMLIARYKMFPEVKEKG
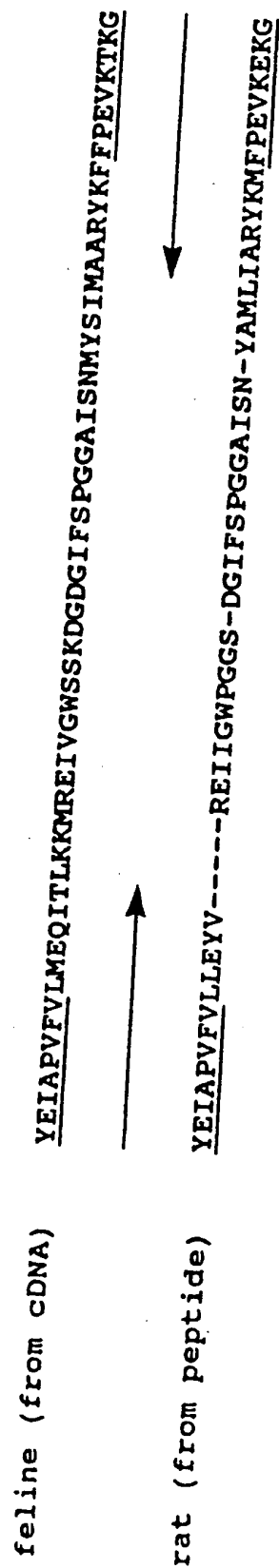
FIG. 1

```
GGGCGGTGCGGGGGTCGAGCCGAAGCAGCTTGCCCGCCAGCCACTCGGAGGCGACCAGCGCCA
            10                30                50
                  M   A   S   P   G   S   G   F   W   S   F   G   S   E   D   G

GACTAGCAGAACCCATGGCCATCTCCGGGCTCTGGCTTTTTGGTCCTTCGGATCTGAAGATG
      70                  90                  110
S   G   D   P   E   N   P   G   T   A   R   A   W   C   Q   V   A   Q   K   F

GCTCTGGGGATCCTGAGAAACCCGGGAACACAGAGCCTGGTGCCAGGTGGCCCAAAAGT
        130                 150                 170
T   G   G   I   G   N   K   L   C   A   L   L   Y   G   D   S   E   K   P   A

TCACGGGCGGCATCGGGAAAACAAGCTATGCGCTCTCTGCTCTACGGAGACTCTGAGAAGCCAG
          190                 210                 230
E   S   G   G   S   V   T   S   R   A   A   T   R   K   V   A   C   T   C   D

CAGAGAGCGGCGGGAGCGTGACCTCGCCGGGGCCGCCACTCGGAAGGTCGCCTGCACCTGTG
            250                 270                 290
Q   K   P   C   S   C   P   K   G   D   V   N   Y   A   L   L   H   A   T   D

ACCAAAAACCCTGCAGCTGCCCCAAAGGAGATGTCAATTATGCACTTCTCCACGCAACAG
          310                 330                 350
L   L   P   A   C   E   G   E   R   P   T   L   A   F   L   Q   D   V   M   N

ACCTGCTGCCAGCCTGTGAAGGAGAAAGGCCCCACTCTCCGCATTTCTGCAAGATGTAATGA
          370                 390                 410
I   L   L   Q   Y   V   V   K   S   F   D   R   S   T   K   V   I   D   F   H

ACATTTTGCTTCAGTACGTGGTGAAAAGTTTTGATAGATCAACTAAAGTGATTGATTTCC
          430                 450                 470
Y   P   N   E   L   L   Q   E   Y   N   W   E   L   A   D   Q   P   Q   N   L

ATTACCCCAATGAGCTTCTTCAAGAGTATAATTGGGAATTGGCAGACCAACCGCAAAATC
          490                 510                 530
E   E   I   L   T   H   C   Q   T   T   L   K   Y   A   I   K   T   G   H   P

TGGAGGAAATTTTGACGCACTGCCAAACAACTCTAAAAATATGGCATTAAAACAGGGCATC
          550                 570                 590
R   Y   F   N   Q   L   S   T   G   L   D   M   V   G   L   A   A   D   W   L

CCCGATATTTTAATCAGCTGTCTACCGGATTGGATATGGTTGGATTAGCAGCAGATTGGT
          610                 630                 650
T   S   T   A   N   T   M   F   T   Y   E   I   A   P   V   F   V   L   L

TGACATCAACAGCAAACACGAACATGTTTACCTATGAGATCGCCCCCTGTATTTGTACTAC
```

FIG.2A

```
                         670                   690                   710
          E   Y   V   T   L   K   K   M   R   E   I   I   G   W   P   G   G   S   G   D
TGGAATATGTGACACTAAAGAAAATGAGGGAAATCATTGGCTGGCCAGGAGGCTCTGGCG
                         730                   750                   770
          G   I   F   S   P   G   G   A   I   S   N   M   Y   A   M   L   I   A   R   Y
ATGGAATCTTTTCTCCTGGTGGTGCCATCTCCAACATGTACGCCATGCTCATTGCCCGCT
                         790                   810                   830
          K   M   F   P   E   V   K   E   K   G   M   A   A   V   P   R   L   I   A   F
ATAAGATGTTTCCAGAAGTCAAGGAAAAGGGGGATGGCGGCTGCCCCAGGCTCATCGCAT
                         850                   870                   890
          T   S   E   H   S   H   F   S   L   K   K   G   A   A   L   G   I   G   T
TCACGTCAGAGCATAGTCACTTTCTCAAGAAGGGAGCTGCAGCCCTTGGGGATCGGAA
                         910                   930                   950
          D   S   V   L   I   K   C   D   E   R   G   K   M   I   P   S   D   L   E
CAGACAGCCGTGATTCTGATTAAATGTGATGAGAGAGGGAAAATGATCCCATCTGACCTTG
                         970                   990                   1010
          R   R   I   L   E   V   K   Q   K   G   F   V   P   F   L   V   S   A   T   A
AAAGAAGAATCCTTGAAGTCAAACAGAAAGGATTTGTTCCTTTCCTGGTGAGTGCCACAG
                         1030                  1050                  1070
          R   R   I   L   E   V   K   Q   K   G   F   V   P   F   L   V   S   A   T   A
AAAGAAGAATCCTTGAAGTCAAACAGAAAGGATTTGTTCCTTTCCTGGTGAGTGCCACAG
                         1090                  1110                  1130
          G   T   T   V   Y   G   A   F   D   P   L   L   A   V   A   D   I   C   K   K
CTGGAACCACTGTGTACGGGGCTTTTGATCCTCTTGGCTGTGGCTGACATCTGCAAAA
                         1150                  1170                  1190
          Y   K   I   W   M   H   V   D   A   W   G   G   G   L   L   M   S   R   K
AATATAAGATCTGGATGCATGTGGATGCTGCTGGGGGTTGGGGAGGGTTACTGATGTCTCGGA
                         1210                  1230                  1250
          H   K   W   K   L   N   G   V   E   R   A   N   S   V   T   W   N   P   H   K
AACACAAGTGGAAGCTGAACGGTGTGGAGAGGGCCAACTCTGTGACATGGAATCCCACA
          M   M   G   V   P   L   Q   C   S   A   L   L   V   R   E   E   G   L   M   Q
AGATGATGGGTGTCCCCTTGCAATGTTCGGCTCTCCTGGTCAGAGAGAGGAGGGACTGATGC
```

FIG. 2B

```
                                                    1270              1290              1310
        S   C   N   Q   M   H   A   S   Y   L   F   Q   Q   D   K   H   Y   D   L   S
        AGAGCTGCAACCAGATGCATGCTTCCTACCTCTTTCAGCAAGATAAGCACTATGACCTGT
                        1330              1350              1370
        Y   D   T   G   D   K   A   L   Q   C   G   R   H   V   D   V   F   K   L   W
        CCTATGACACGGGAGACAAGGCCTTGCAGTGTGGACGCCACGTCGATGTCTTTAAATTAT
                        1390              1410              1430
        L   M   W   R   A   K   G   T   G   F   E   A   H   I   D   K   C   L   E
        GGCTCATGTGGAGAGCAAAGGGGACTACTGGATTTGAAGCTCACATTGATAAGTGTTTGG
                        1450              1470              1490
        L   A   E   Y   L   Y   N   I   I   K   N   R   E   G   Y   E   M   V   F   D
        AGCTGGCAGAGTATTTATACAATATCATTAAAAACCGAGAAGGATATGAAATGGTGTTCG
                        1510              1530              1550
        G   K   P   Q   H   T   N   V   C   F   W   F   V   P   P   S   L   R   V   L
        ATGGGAAGCCTCAGCACACAAATGTCTGCTTCTGGTTTGTACCTCCTAGTTTGCGAGTTC
                        1570              1590              1610
        E   D   N   E   E   R   M   S   R   L   S   K   V   A   P   V   I   K   A   R
        TGGAAGACAATGAAGAGAGAATGAGCCGCCTCTCAAAGGTGGCGCCAGTGATTAAAGCCA
                        1630              1650              1670
        M   M   E   Y   G   T   T   M   V   S   Y   Q   P   L   G   D   K   V   N   F
        GAATGATGGAGTATGGGACCACAATGGTCAGCTACCAACCCTTAGGAGATAAGGTCAACT
                        1690              1710              1730
        F   R   M   V   I   S   N   P   A   T   H   Q   D   I   D   F   L   I   E
        TCTTCCGCATGGTCATCTCAAACCCTGCAGCAACTCACCAAGACATTGACTTCCTCATTG
                        1750              1770              1790
        E   I   E   R   L   G   Q   D   L   *
        AAGAAATCGAACGCCTGGGACAAGATTTGTAATCACTTTGCTCACCAAACTTTCAGTTCT
                        1810              1830              1850
        CTAGGTAGACAGCTAAGTTGTCACAAACTGTGTAAATGTATTTGTAGTTTGTTCCAGAGT
                        1870              1890              1910
        AATTCTATTTCTATATCGTGGTGTCACAGTAGAGTCCAGTTTAAAA
                        1930              1950
```

FIG. 2C

```
                                                    M   A   S
agctcgcccgcagctcgcactcgcaggcgacctgctccagtctccaaagccgatggcatc
              10                  30                  50

 P   G   S   G   F   W   S   F   G   S   E   D   G   S   G   D   S   E   N   P
tccgggctctggcttttggtcttcggtcggaagatggctctgggtcggattccgagaatcc
              70                  90                  110

 G   T   A   R   A   W   C   Q   V   A   Q   K   F   T   G   I   G   N   K
cggcacagcgcgagcctggtgtgccaagttcacggggcatcggaaacaa
              130                 150                 170

 L   C   A   L   L   Y   G   D   A   E   K   P   A   E   S   G   G   S   Q   P
actgtgccctgctctacggagacgccgagaagccggagagccggggagccaacc
              190                 210                 230

 P   R   A   A   R   K   A   A   C   A   C   D   Q   K   P   C   S   C   S
cccgcggccgccgcgaaggccgcctgcgcctgcgaccagaagccctgctctgctc
              250                 270                 290

 K   V   D   V   N   Y   A   F   L   H   A   T   D   L   L   P   A   C   D   G
caagtggatgtcaactacgcgttcctccatgcaacagacctgctgccgtgtgatgg
              310                 330                 350

 E   R   P   T   L   A   F   L   Q   D   V   M   N   I   L   L   Q   Y   V   V
agaaaggcccactttggcgtttctgcaagatgttatgaacattttacttcagtatgtggt
              370                 390                 410

 K   S   F   D   R   S   T   K   V   I   D   F   H   Y   P   N   E   L   L   Q
gaaaagtttcgatagatcaaccaaagtgattgatttccattatcctaatggcttctcca
              430                 450                 470

 E   Y   N   W   E   L   A   D   Q   P   Q   N   L   E   E   I   L   M   H   C
agaatatattggaattggcagacccacaacaaaatttggaggaaattttgatgcattg
              490                 510                 530

 Q   T   T   L   K   Y   A   I   K   T   G   H   P   R   Y   F   N   Q   L   S
ccaaacaactctaaaatatgcaattaaaacagggcatcctagatacttcatcaacttc
              550                 570                 590

 T   G   L   D   M   V   G   L   A   D   W   L   T   S   T   A   N   T   N
tactggtttggatatggttggattagcagcagactggctgacatcaacagcaaatactaa
```

FIG. 3A

```
          610            630            650
   M  F  T  Y  E  I  A  P  V  F  V  L  L  E  Y  V  T  L  K  K
catgttcacctatgaaattgctccagtatttgtgctttggaatatgtcacactaaagaa
                 670            690            710

   M  R  E  I  I  G  W  P  G  G  S  G  D  G  I  F  S  P  G  G
aatgagagaaatcattggcctgctggcgatgggatatttctcccggtgg
      730            750            770

   A  I  S  N  M  Y  A  M  M  I  A  R  F  K  M  F  P  E  V  K
cgccatatctaacatgtatgccatgatgatcgcacgcttaagatgttccagaagtcaa
            790            810            830

   E  K  G  M  A  A  L  P  R  L  I  A  F  T  S  E  H  S  H  F
ggagaaaggaatggctgctcctcccaggctcattgccttcacgtctgaacatagtcattt
            850            870            890

   S  L  K  K  G  A  A  A  L  G  I  G  T  D  S  V  I  L  I  K
ttctctcaagaaggggagctgcagcctaggattggaacagacagcgtgattctgattaa
            910            930            950

   C  D  E  R  G  K  M  I  P  S  D  L  E  R  R  I  L  E  A  K
atgtgatgagagaggaaaatgattccatctgatcttgaaaagaattcttgaagccaa
            970            990            1010

   Q  K  G  F  V  P  F  F  L  V  S  A  T  A  G  T  T  V  Y  G  A
acagaaagggtttgttcctttcttcctcgtgagtgccacagctggaaccacgtgtacggagc
            1030           1050           1070

   F  D  P  L  L  A  V  A  D  I  C  K  K  Y  K  I  W  M  H  V
atttgacccccctcttagctgctgacattgcaaaagtataagatctggatgcatgt
            1090           1110           1130

   D  A  W  G  G  G  L  L  M  S  R  K  H  K  W  K  L  S  G
ggatgcagcttgggggtggggggattactgatgtcccgaaacacaagtggaaactgagtgg
            1150           1170           1190

   V  E  R  A  N  S  V  T  W  N  P  H  K  M  M  G  V  P  L  Q
cgtggagagggccaactctgtgacgtggaatccacacaagatgatgggggtccctttgca
```

FIG.3B

```
                        1230                        1250
      C   S   A   L   L   V   R   E   E   G   L   M   Q   N   C   N   Q   M   H   A
      gtgctctgctcctgccggttagagaagaggggattgatgcagaattgcaaccaaatgcatgc
                        1270                        1290                        1310
      S   Y   L   F   Q   Q   D   K   H   Y   D   L   S   Y   D   T   G   D   K   A
      ctcctacctctttcagcaagataaacattagacctgtcctatgacactggagacaaggc
                        1330                        1350                        1370
      L   Q   C   G   R   H   V   D   V   F   K   L   W   L   M   W   R   A   K   G
      cttacagtgcgggacgccacgttgatgtttaaactatggctgatgtggaggggcaaaggg
                        1390                        1410                        1430
      T   T   G   F   E   A   H   V   D   K   C   L   E   L   A   E   Y   L   Y   N
      gactaccgggtttgaagcgcatgttgataaatgttggcagagtattatacaa
                        1450                        1470                        1490
      I   I   K   N   R   E   G   Y   E   M   V   F   D   G   K   P   Q   H   T   N
      catcataaaaaaccgagagaggatatgagatatggtgtttgatgggaagcctcagcacacaa
```

FIG. 3C

```
          1510                1530                1550
  V   C   F   W   Y   I   P   P   S   L   R   T   L   E   D   N   E   R   M
 tgtctgctttctgtgtacattcctccaagcttgccgtactctggaagacaatgaagagagaat
          1570                1590                1610
  S   R   L   S   K   V   A   P   V   I   K   A   R   M   M   E   Y   G   T   T
 gagtcgcctctcgaaggtgctccagtggctcaagataatgatggagtatggaaccac
          1630                1650                1670
  M   V   S   Y   Q   P   L   G   D   K   V   N   F   F   R   M   V   I   S   N
 aatggtcagctaccaacccctgggagacaaggtcaatttcttccgcatggtcatctcaaa
          1690                1710                1730
  P   A   A   T   H   Q   D   I   D   F   L   I   E   E   I   E   R   L   G   Q
 cccagcggcaactcaccagacattgacttcctgattgaagaaatagaacgccctggaca
          1750                1770                1790
  D   L   *
 agatttataataaccttgctcaccagctgttccacttctctaggtagacaattaagttg
          1810                1830                1850
 tcacaaactgtgtgaatgtattgtagtttgttccaaagtaaatctatttctatattgtg
          1870                1890                1910
 gtgtcaaagtagagtttaaaattaaacaaaagacattgctcctttaaaagtcccttt
          1930                1950                1970
 cttaagtttagaataacctctctaagaattcgtgacaaaaggctatgttctaatcaataag
          1990                2010                2030
 gaaaagcttaaaattgttataatacttcccttactttaatatagtgtgcaaagcaaac
          2050                2070                2090
```

FIG. 3D

```
        GAP WEIGHT:    3.000      AVERAGE MATCH:     0.540
     LENGTH WEIGHT:    0.100   AVERAGE MISMATCH:    -0.396

          QUALITY:   856.2            LENGTH:       585
            RATIO:   1.464              GAPS:         0
PERCENT SIMILARITY:  97.436   PERCENT IDENTITY:   96.068

   4817,  PEP HGT2.PEP              AUGUST 22, 1990 08:20  ..
```

```
  1 MASPGSGFWSFGSEDGSGDPENPGTARAWCQVAQKFTGGIGNKLCALLYG  50
    |||||||||||||||||||•|||||||||||||||||||||||||||||||
  1 MASPGSGFWSFGSEDGSGDSENPGTARAWCQVAQKFTGGIGNKLCALLYG  50

 51 DSEKPAESGGSVTSRAATRKVACTCDQKPCSCPKGDVNYALLHATDLLPA 100
    |•|||||||||  ••|||•||•||•|||||||||•|•|||||:|||||||||
 51 DAEKPAESGGSQPPRAAARKAACACDQKPCSCSKVDVNYAFLHATDLLPA 100

101 CEGERPTLAFLQDVMNILLQYVVKSFDRSTKVIDFHYPNELLQEYNWELA 150
    |:|||||||||||||||||||||||||||||||||||||||||||||||||
101 CDGERPTLAFLQDVMNILLQYVVKSFDRSTKVIDFHYPNELLQEYNWELA 150

151 DQPQNLEEILTHCQTTLKYAIKTGHPRYFNQLSTGLDMVGLAADWLTSTA 200
    |||||||||| |||||||||||||||||||||||||||||||||||||||
151 DQPQNLEEILMHCQTTLKYAIKTGHPRYFNQLSTGLDMVGLAADWLTSTA 200

201 NTNMFTYEIAPVFVLLEYVTLKKMREIIGWPGGSGDGIFSPGGAISNMYA 250
    ||||||||||||||||||||||||||||||||||||||||||||||||||
201 NTNMFTYEIAPVFVLLEYVTLKKMREIIGWPGGSGDGIFSPGGAISNMYA 250

251 MLIARYKMFPEVKEKGMAAVPRLIAFTSEHSHFSLKKGAAALGIGTDSVI 300
    |:|||:|||||||||||||:|||||||||||||||||||||||||||||||
251 MMIARFKMFPEVKEKGMAALPRLIAFTSEHSHFSLKKGAAALGIGTDSVI 300

301 LIKCDERGKMIPSDLERRILEVKQKGFVPFLVSATAGTTVYGAFDPLLAV 350
    |||||||||||||||||||||•|||||||||||||||||||||||||||||
301 LIKCDERGKMIPSDLERRILEAKQKGFVPFLVSATAGTTVYGAFDPLLAV 350

351 ADICKKYKIWMHVDAAWGGGLLMSRKHKWKLNGVERANSVTWNPHKMMGV 400
    |||||||||||||||||||||||||||||||||•||||||||||||||||
351 ADICKKYKIWMHVDAAWGGGLLMSRKHKWKLSGVERANSVTWNPHKMMGV 400
```

FIG.4A

```
401  PLQCSALLVREEGLMQSCNQMHASYLFQQDKHYDLSYDTGDKALQCGRHV  450
     ||||||||||||||||||·|||||||||||||||||||||||||||||||
401  PLQCSALLVREEGLMQNCNQMHASYLFQQDKHYDLSYDTGDKALQCGRHV  450

451  DVFKLWLMWRAKGTTGFEAHIDKCLELAEYLYNIIKNREGYEMVFDGKPQ  500
     ||||||||||||||||||||:|||||||||||||||||||||||||||||
451  DVFKLWLMWRAKGTTGFEAHVDKCLELAEYLYNIIKNREGYEMVFDGKPQ  500

501  HTNVCFWFVPPSLRVLEDNEERMSRLSKVAPVIKARMMEYGTTMVSYQPL  550
     ||||||:::|||||·||||||||||||||||||||||||||||||||||
501  HTNVCFWYIPPSLRTLEDNEERMSRLSKVAPVIKARMMEYGTTMVSYQPL  550

551  GDKVNFFRMVISNPAATHQDIDFLIEEIERLGQDL  585
     ||||||||||||||||||||||||||||||||||
551  GDKVNFFRMVISNPAATHQDIDFLIEEIERLGQDL  585
```
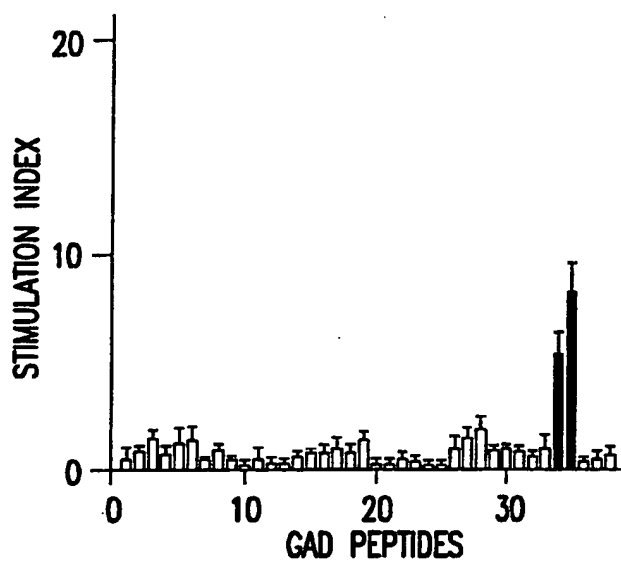
FIG.4B
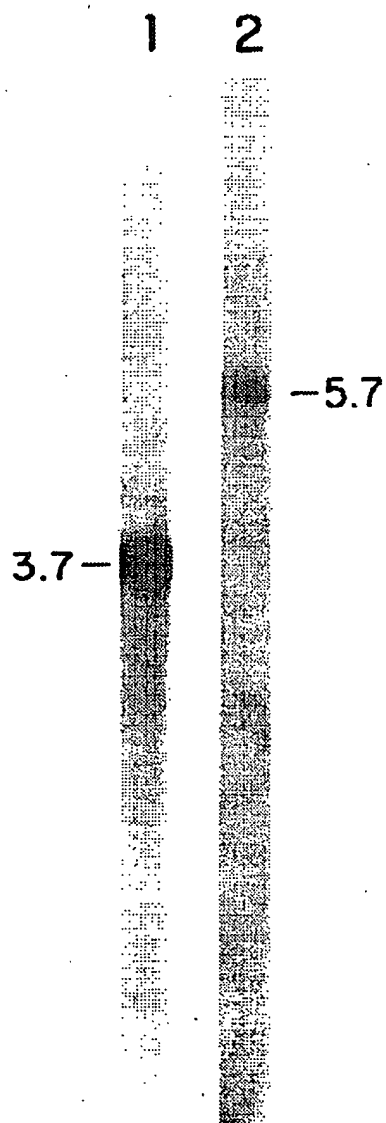
FIG.5
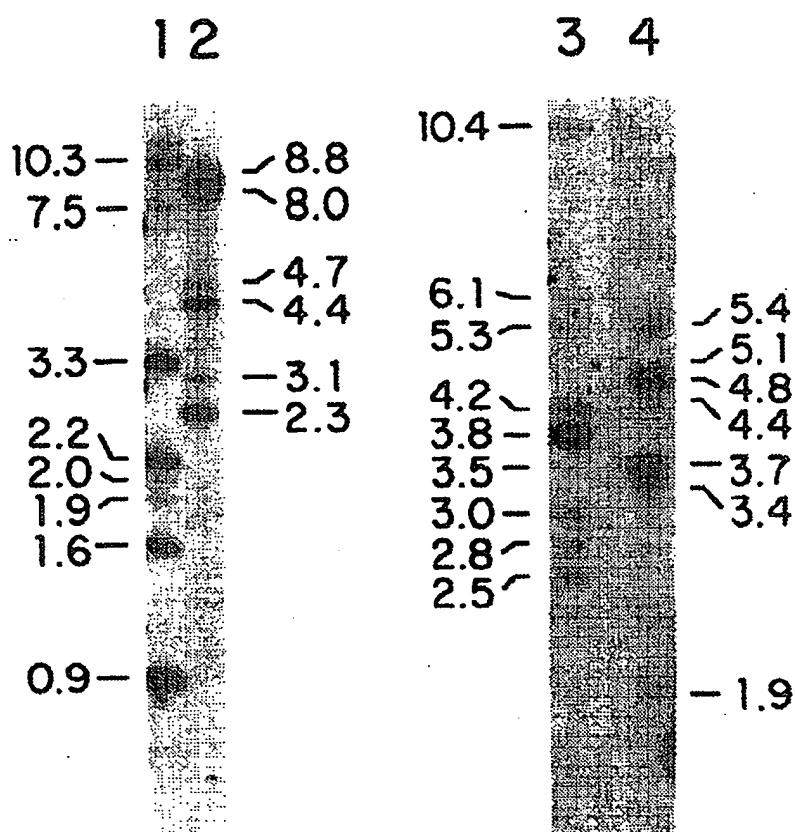
FIG.6
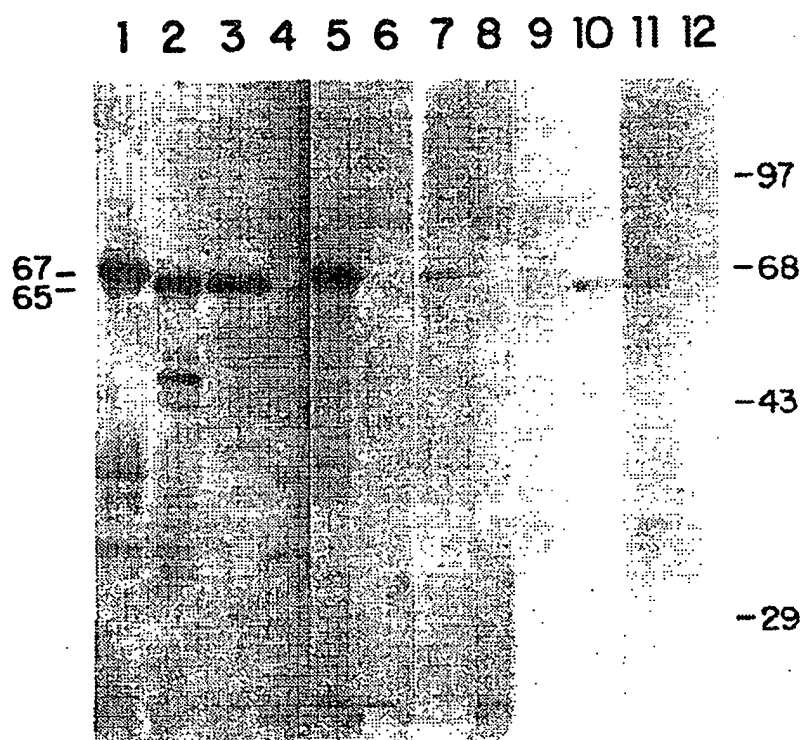
FIG.7
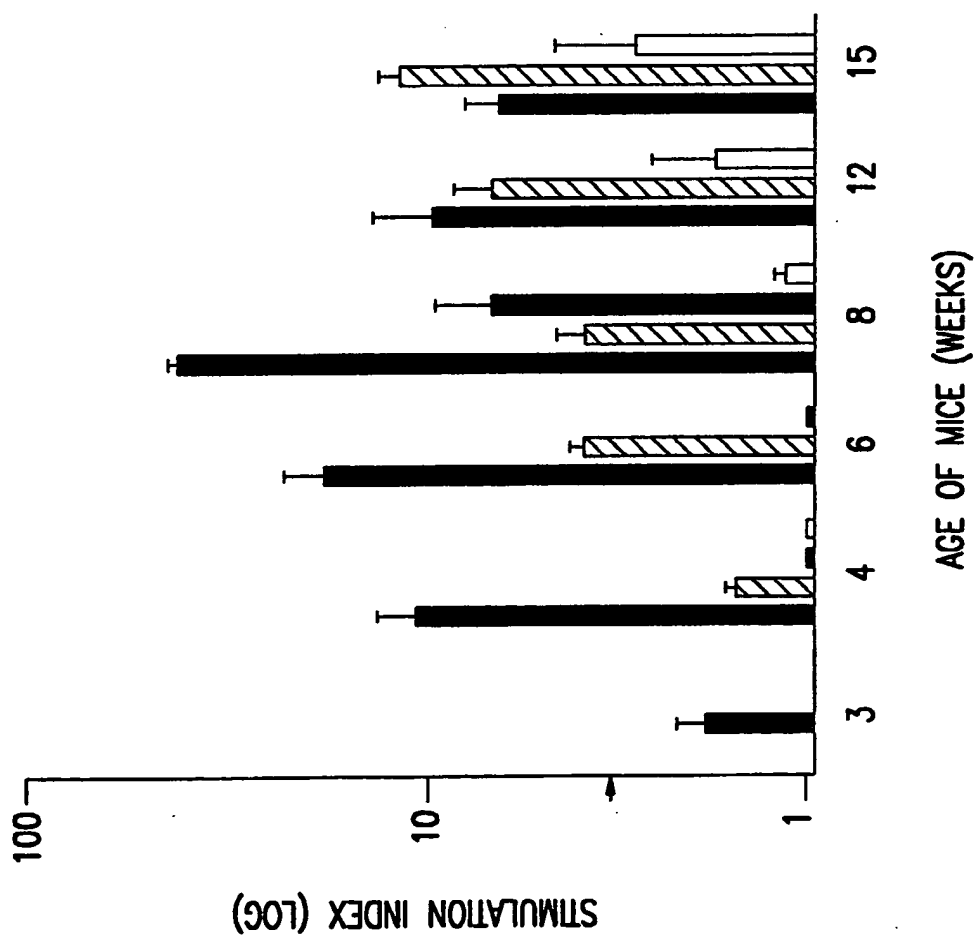
FIG.8
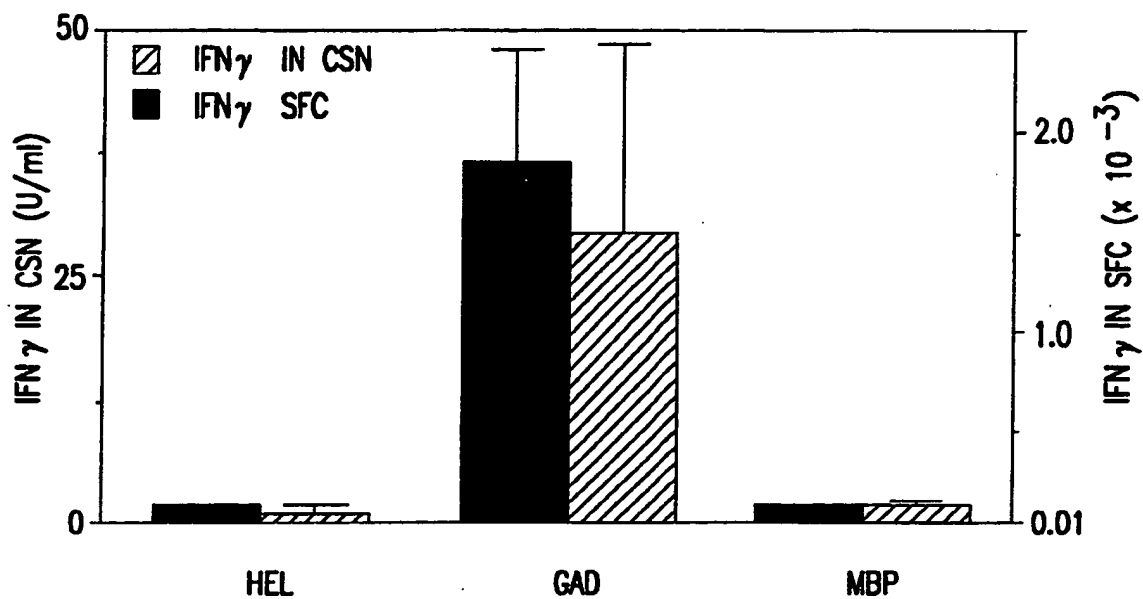
FIG.9A
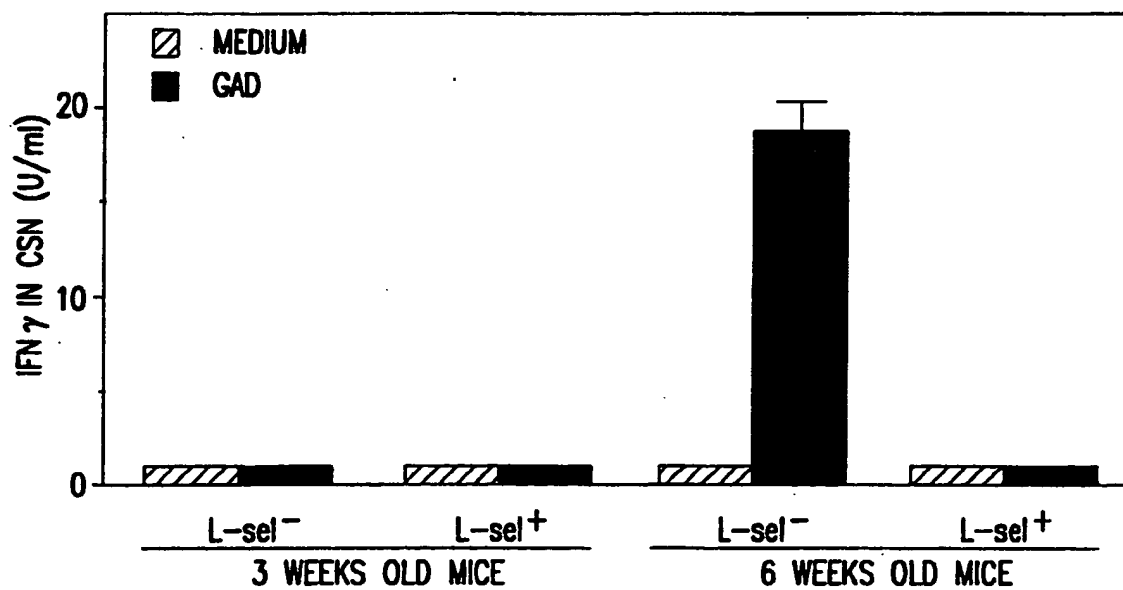
FIG.9B

FIG.10A

FIG.10B

FIG.10C

FIG.11